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THE CYCLIC NATURE AND MAGNITUDE OF CELL DIVISION IN GASTRIC MUCOSA OF URODELE LARVAE REARED IN THE POND AND LABORATORY

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In a previous publication, Scheving, Chiakulas and Abzug (1959) reported that in salamander larvae the epidermal mitotic rate in animals from two different environments was characteristically cyclic over a 24-hour period. Although the cycle was manifest and corresponded as to time in both groups, the over-all mean mitotic rate of the epidermis in animals collected from the natural pond environment was 20 times that in the epidermis of laboratory-reared animals. A number of differences in the ecological conditions of the pond and laboratory environments were presented as possible factors responsible for the differential in the mitotic rates of the two groups. The results of our study on epidermis bring up a number of questions: (1) Do these ecological factors, possibly responsible for the differences in the mitotic rates, act specifically on the epidermis alone, since this tissue is directly exposed to the external environment, or do they act in an unspecific indirect manner so as to affect the general physiology of the animal and thereby affect the mitotic rate of other tissues as well? (2) Is the 24-hour cycle, characteristic of the mitotic rate of epidermis, present in other tissues?

To furnish possible answers to these questions, it was decided to study the magnitude and cyclic nature of the mitotic rate of the gastric mucosal epithelium of the same two groups of animals, pond and laboratory, used previously for the epidermal mitotic rate determinations. If a difference existed between the magnitude of mitosis in the two groups, a general physiological effect of environmental factors might be deduced. Other investigators (Klein and Geisel, 1947; Leblond and Stevens, 1948) have indicated that gut mucosal epithelium in the rat shows no significant cyclic variations in mitotic rate but that this tissue has a constant renewal rate. Therefore, it was of interest to determine if the phenomenon of daily cyclic variation in the mitotic rate of gastric mucosal epithelium existed in salamander larvae, and if so, what similarities or differences existed between the cycles of gastric mucosal epithelium and epidermis. The results of the determinations on the mitotic rate of gastric mucosal epithelium are the basis of this report.

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Materials and Methods

Two groups of *Amblystoma punctatum* larvae, which were used in an earlier study of the epidermal mitotic rate (Scheving *et al.,* 1959), were used in the present study. The two groups were: (1) "laboratory" animals, which were reared in the laboratory from the egg stage until the chronological age of 6 weeks after hatching, and (2) "pond" animals, which were collected as larvae from the pond and which were approximately at the same stage of development as the laboratory animals when sacrificed. The differences in the two environments cannot be rigidly defined since no control procedures were used. It may be stated, however, that the laboratory animals were reared in city tap water which was run through commercial filters to remove the chlorine. In contrast, the pond animals grew in the semi-stagnant water of the natural pond. During the 24-hour collection period, water temperatures were taken hourly, and the temperature of the pond water ranged between 16° C. and 21° C. The laboratory animals were raised from the egg stage at heated room temperature, ranging from 20° C. to 24° C. During the 24-hour period of sacrifice, the room temperature averaged 21° C. Other possible differences between the two environments, such as differences in the oxygen tension and salt concentration of the water, degree of activity of the animals, were not determined.

Table I

Mean mitotic indices (mitoses/1000 cells) with standard deviations of gastric mucosal epithelium

Hour	Laboratory animals	Pond animals
6:30 A.M.	0.11 ± 0.07	2.89 ± 0.44
8:30 A.M.	0.33 ± 0.12	2.89 ± 0.69
10:30 A.M.	1.21 ± 0.40	5.65 ± 0.80
12:30 P.M.	1.09 ± 0.66	6.01 ± 1.62
2:30 P.M.	0.88 ± 0.35	4.60 ± 0.61
4:30 P.M.	0.97 ± 0.34	4.32 ± 1.11
6:30 P.M.	0.38 ± 0.40	4.14 ± 0.81
8:30 P.M.	0.31 ± 0.13	4.40 ± 0.85
10:30 P.M.	0.35 ± 1.14	4.60 ± 1.42
12:30 A.M.	0.41 ± 0.15	5.24 ± 0.92
2:30 A.M.	0.50 ± 0.12	7.02 ± 1.23
4:30 A.M.	0.43 ± 0.18	3.90 ± 0.63
Over-all Daily Mean	0.58 ± 0.34	4.64 ± 0.93

Groups of 20 animals from each environment were sacrificed every two hours over a 24-hour period, fixed in Bouin's, sectioned transversely and stained with iron hematoxylin. Nuclear and mitotic counts were made on the gastric mucosal epithelium. Only the cells lining the lumen of the stomach were included in the counts; areas of continuity of the mucosal epithelium with the gastric glands were excluded. To avoid duplication, the number of nuclei and mitotic figures was recorded for every third section. At least 5000 nuclei were counted in each specimen. The mitotic index was calculated as the number of mitoses per 1000 cells. The mean mitotic indices for each time-period group and the mean over-all mitotic indices for the 24-hour periods, for both laboratory and pond animals, were determined and are indicated in Table I. Statistical comparisons and analyses were made by computing the standard error of the differences between two means and the consequent probability.

RESULTS

The mean mitotic indices of the gastric mucosal epithelium for each of the 12 time periods over a 24-hour day and for both animal groups are listed in Table I. When these indices are plotted in a graph (Figs. 1, 2), it is obvious that the mucosal mitotic activity of both the laboratory and pond animals is characteristically cyclic in nature.

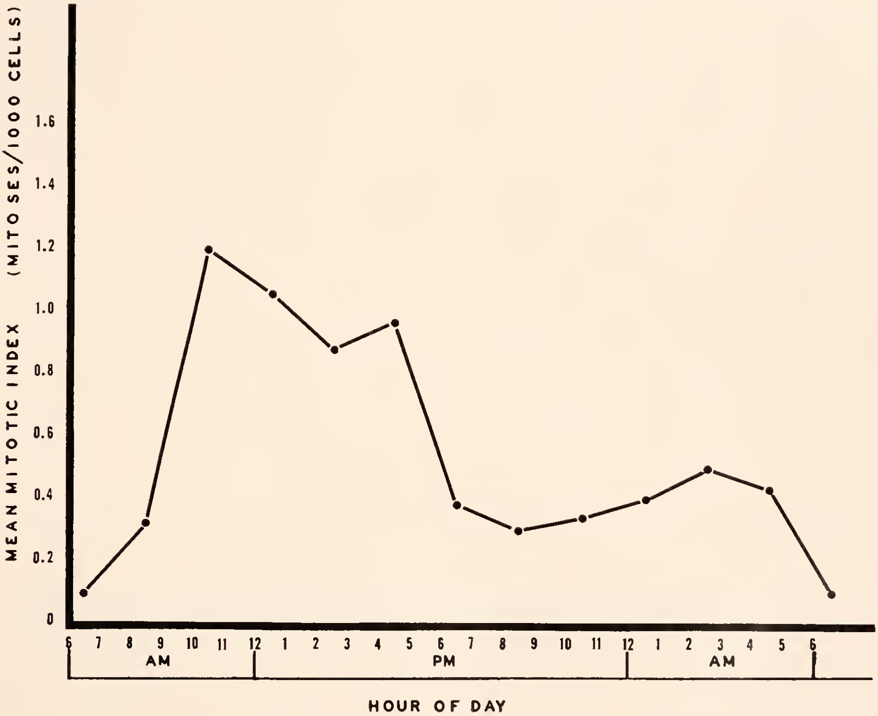

FIGURE 1. Mean indices, at two-hour intervals over a 24-hour period, for mitotic rate of gastric mucosal epithelium of laboratory animals.

In the laboratory animals the peak hour of activity is at 10:30 A.M., at which time the mean mitotic index is 1.21 ± 0.40 (Table I; Fig. 1). Following this peak, a gradual decrease in the number of dividing cells is recorded until 4:30 P.M., when the daily over-all mean mitotic index of 0.58 ± 0.34 is approached. Subsequently, there is a relatively rapid decrease in mitotic activity and the mitotic indices are below the 24-hour mean index during a seemingly stable period from 6:30 P.M. through 6:30 A.M., with the exception of a minor increase at 2:30 A.M.

The mitotic index of the 10:30 A.M. peak was compared with the over-all mean mitotic rate and the difference was found to be statistically significant ($0.001 < p < 0.01$). When the 10:30 A.M. peak index was compared to the 6:30 A.M.

minimum index, the difference indicated greater statistical significance ($p < 0.001$). It also can be seen from Table I and Figure 1 that the mitotic rate at 10:30 A.M. is twice the average rate for all hours and 10 times the minimum rate recorded at 6:30 A.M. The minor rise in the rate of cellular division observed at 2:30 A.M. has no statistical significance.

In the pond animals, two peaks of high cellular division occur—one at 12:30 P.M. when the mean mitotic index reaches the value of 6.01 ± 1.62, and another at 2:30 A.M. when the mean mitotic index of 7.02 ± 1.23 is recorded (Table I; Fig. 2). When these peak values are compared to the daily over-all mean mitotic index of 4.64 ± 0.93, they are not statistically significant. However, when these two values are compared to the minimum index of 2.89 ± 0.44 occurring at 6:30 A.M., both the differences are statistically significant ($p < 0.01$).

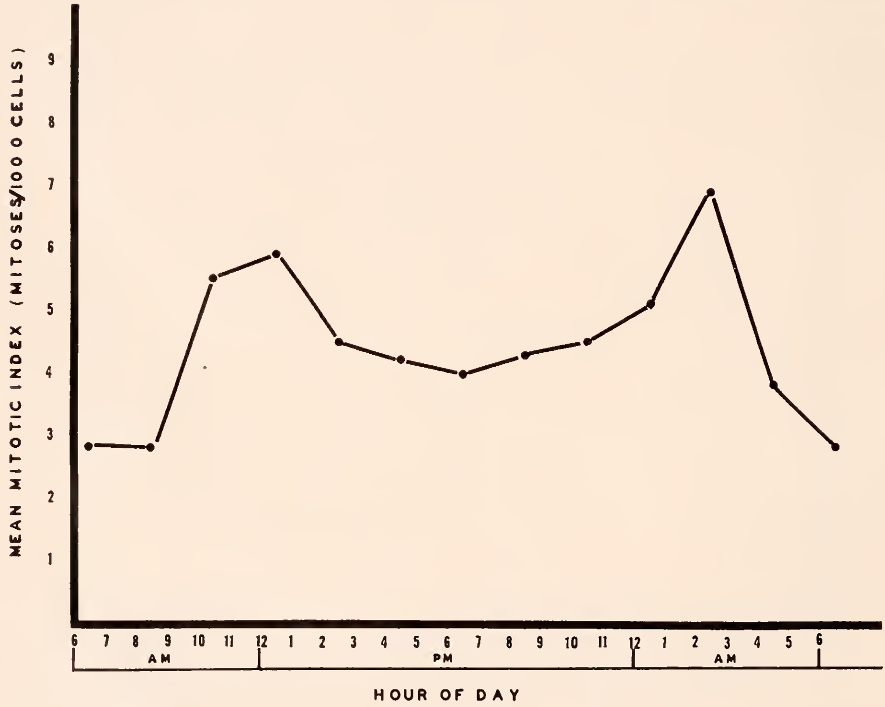

FIGURE 2. Mean indices, at two-hour intervals over a 24-hour period, for mitotic rate of gastric mucosal epithelium of pond animals.

When the mitotic indices of both laboratory and pond animals are plotted on the same scale (Fig. 3) a number of interesting observations are made. It is evident from the graphs (Fig. 3) that the rate of cell division follows a similar cyclic pattern in both groups, with a high period of activity between 10:30 A.M. and 12:30 P.M. The 2:30 A.M. peak present in the pond group is not present in the laboratory group, although it is probable that the slight rise at 2:30 A.M. above the preceding period may represent a rather feeble acceleration corresponding to the peak present at this time in the pond group. Minimum mitotic activity is established in both environmental groups between the hours of 6:30 A.M. and 8:30 A.M.

The most striking difference in the two animal groups is in the magnitude of the mitotic rates. The over-all mean mitotic rate in the gastric mucosa of the pond animals is approximately 8 times that of the laboratory animals (see Table I; compare two graphs of Figure 3).

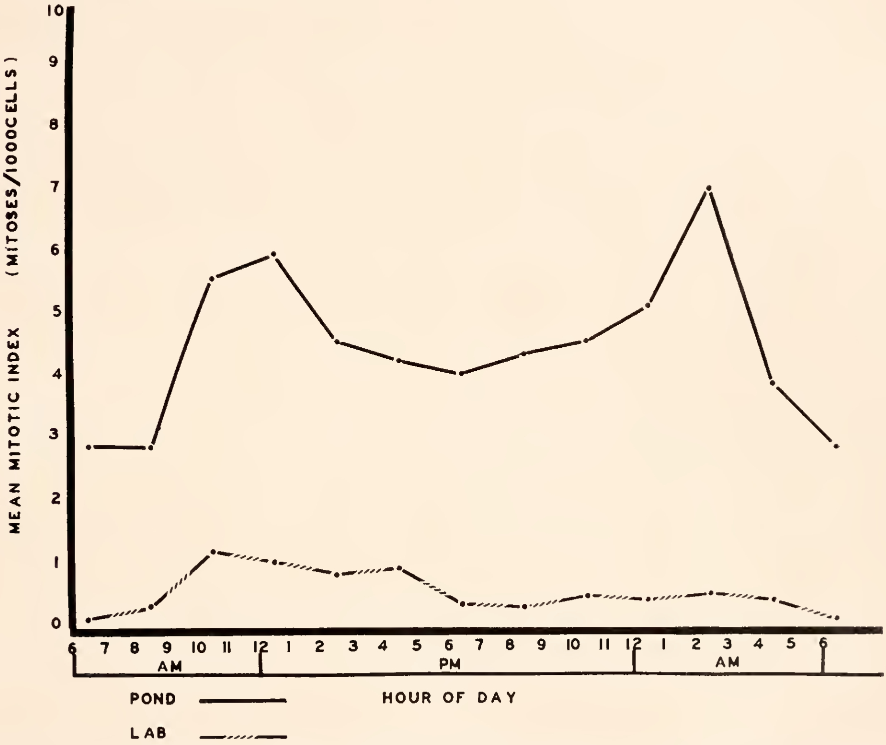

FIGURE 3. Mean indices of mucosal mitotic rate of laboratory and pond animals plotted on the same scale.

DISCUSSION

It is not the intent of this report to review the rapidly accumulating literature dealing with the cyclic nature of the rate of mitosis in various tissues or on the periodicity observed in many other biological processes. Brief summaries or reviews of some of the pertinent investigations in this field have been made by Scheving (1959), Leedale (1959) and Harker (1958). Here, we will discuss only the significance and implications of our results and relate and compare these results to those obtained in a similar investigation of the mitotic rate in urodele larval epidermis (Scheving *et al.*, 1959).

The results of this investigation, first of all, clearly demonstrate that the mitotic rate of the gastric mucosal epithelium is cyclic in character with occurrence of typical peaks and lows in the rate of cellular division during the 24-hour period.

Secondly, this finding indicates that gut mucosa is not a tissue with a constant replacement rate, at least in urodele larvae, and therefore this finding differs from the conclusions of Klein and Geisel (1947), and Leblond and Stevens (1948) that gut mucosal epithelium in the rat demonstrated no significant periodic fluctuations in its mitotic rate. Although a great number of previous investigations have dealt with periodicity of mitotic rate of epidermis or cornea, our results add to the evidence that other tissues may have similar cycles. What the basic factor underlying the cause of this cyclic nature of the cellular division rate is remains unanswered. However, whatever the cause, whether it be an intrinsic characteristic of the cells themselves or an externally imposed factor, it is apparent that it is operative in several tissues simultaneously.

When the graphs of Figure 3 are compared, it is noted that although the mucosal mitotic rate of both pond and laboratory animals is cyclic, the over-all daily mitotic rate of the pond group is 8 times that of the laboratory animals' rate. A similar but greater differential was reported for the epidermal mitotic rates of the same animals (Scheving *et al.*, 1959). Again, the reason for this difference cannot be explained at the present time. In the report on epidermis, a number of differences in the ecological factors of the pond and laboratory environments—such as oxygen tension, activity, diet, salt concentration—were mentioned as possible causes for the differences in mitotic rates between the two groups of animals. The implication was that these factors acted directly on the epidermis, since it was exposed to the external environment, or acted in some way to increase the general growth rate of the animals. Since this differential in mitotic rate is present also in the mucosal epithelium of the same animals, it appears that whatever the factors are that operate in producing this increased mitotic rate in pond over laboratory animals, they act in general to result in an enhanced rate of growth. However, this inference does not preclude the possibility that some of the ecological factors may still be acting directly on the epidermal tissue, because the gastric mucosal rate of the pond animals is only 8 times that of the laboratory animals, whereas the pond epidermal rate is 20 times that of the laboratory rate. Also, when it is considered that for the 24-hour period the mean mucosal mitotic index and the epidermal mitotic index of the laboratory animals are approximately the same, it is seen that the epidermis of the pond animals responds to a greater degree than does the mucosal epithelium. This is so probably because the epidermis is directly exposed to the environmental factors. In contrast, the mucosal epithelium, which is not directly exposed to the external environment, shows less of a mitotic response.

No correlation could be seen between the water temperature and the actual mitotic rate for specific hours. In the pond, the high temperature of 21° C. was recorded between 12:00 noon and 1:00 P.M. Correspondingly, a peak with an index of 6.01 ± 1.62 was seen in the mitotic cycle at 12:30 P.M. However, the highest peak in the cycle, with an index of 7.02 ± 1.23, occurred at 2:30 P.M. when the pond water temperature was 16.5° C. The cyclic nature of the mitotic rate thus appears to be unaffected by fluctuations in temperature over the 24-hour period. However, the mean mitotic rate over the 24 hours may be affected by the average temperature. The laboratory animals were raised at room temperature averaging 22° C. In contrast, the pond animals were subjected to colder temperatures in the pond during their period of growth, and on the day of collection the average pond temperature was only 18.5° C. Although pond and laboratory temperatures overlapped during some hours of the period over which the animals were sacrificed, at no time did the mitotic rates of the two groups show any overlap in magnitude.

The results of this investigation again point to the importance of knowing the 24-hour cyclic variations in the mitotic rates of tissues. When mitotic rates are used to interpret the results of experimental procedures, it is necessary to know if the animals involved were sacrificed at the same hours. Otherwise, comparisons and correlations or conclusions based on mitotic indices may be invalid. It is also of importance to know the conditions under which animals are reared, since differences in the environmental factors may cause great differences in the over-all mitotic rate of the tissues. Although in this investigation determinations were not made to establish the actual degree of difference between the pond and laboratory environments in terms of oxygen tension and salt concentration of the water, temperature, diet and animal activity, these differences may be responsible for the differentials seen between the mitotic rates of the two groups of animals. Controlled experiments are in progress to test the effect of each individual factor on mitotic rate of tissues.

SUMMARY

1. The mitotic rate of gastric mucosal epithelium in both pond- and laboratory-reared urodele larvae is cyclic over a 24-hour period. Maximum rate of cell division occurs in both groups between the hours of 10:30 A.M. and 12:30 P.M.; minimum mitotic activity is recorded between 6:30 A.M. and 8:30 A.M.

2. In pond animals, the mitotic rate of gastric mucosa is 8 times the rate of the mucosa of laboratory-reared animals. The environmental differences between pond and laboratory are suggested as the underlying cause for the differential in the rates of mitosis in the two groups of animals. Since a similar difference was previously found in the epidermal mitotic rates of the same animals, it is suggested that in the pond environment an enhanced rate of growth is present.

3. The difference between the pond and laboratory mucosal daily mean mitotic rate is of lesser magnitude than the difference between the epidermal rates of the same animals. This fact indicates that the epidermis may be responding directly to exogenous environmental factors, whereas the mucosa responds only to a lesser degree since it is not directly exposed to the environmental factors.

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X-RAY EFFECTS ON SEX OF PROGENY IN SCIARA COPROPHILA

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Sciara coprophila characteristically gives "unisexual" progenies; *i.e.,* the offspring from any pair mating are practically all of the same sex, either males or females. This "sex of progeny" is determined by the sex chromosome constitution of the female (Moses and Metz, 1928). Genetically there are two kinds of sex chromosomes, designated X and X′ (prime). Females are either XX or XX′; the former are male-producers, the latter female-producers (Metz and Moses, 1928). Sciara males all have the same sex chromosome constitution, form only one type of sperm, and ordinarily exert no influence on the sex ratio of their progeny. The irradiation studies presented here reveal the conditions under which the male *can* influence the sex ratio of his progeny, and thereby they clarify the relationship between "sex of progeny" and the determination of sex of the individual.

All Sciara zygotes begin development with the same chromosome complement. This complement ordinarily includes three sex chromosomes, two of which are identical sister halves of the X derived from the sperm; the third one is an X or X′ derived from the egg. At the seventh or eighth cleavage an elimination of sex chromosomes occurs in the somatic nuclei: in the production of female embryos one paternally-derived X is eliminated, in the production of male embryos both are eliminated; thus the female somatic complement comes to include two sex chromosomes, the male somatic complement only one. In the embryonic germ line the three sex chromosomes are retained until after the germ cells have migrated to the definitive gonad site; then, in both sexes alike, a paternal X is eliminated, leaving two sex chromosomes in the differentiated germ line. This pattern of sex chromosome behavior indicates that sex of the individual fly is not determined prior to chromosome elimination.

The strain of flies used in the experiments reported here produced an exceedingly low number of exceptional males (males among the progeny of female-producers) and exceptional females (females among the progeny of male-producers); progeny counts on one hundred normal females bred to normal males yielded only 0.1% exceptional sex types. A number of conditions are known which may increase the percentage of exceptional sexes. An analysis of these cases (Metz and Schmuck, 1931; Reynolds, 1938; Crouse, 1960) shows the following to be most likely: (1) that the XX-X′ mechanism controls not the sex of the progeny *per se* but rather the type of chromosome elimination which takes place in the embryonic somatic nuclei at the seventh or eighth cleavage; (2) that sex of the individual fly is governed by the somatic complement; and (3) that sex differentiation follows the pattern set by the somatic complement even when the germ line contains an irregular number of sex chromosomes.

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[1] The studies reported here were supported by grants G-6176 and G-9682 from the National Science Foundation.

In the experimental studies cited above (Reynolds, 1938; Crouse, 1960) cases of nondisjunction of sex chromosomes in the female germ line were analyzed: when the zygote received two sex chromosomes from the egg, the type of chromosome elimination in the embryonic soma was strictly in accordance with the XX-XX′ mechanism. That is to say, the exceptional sex types arose *not* because of errors in chromosome elimination but rather in accordance with the sex chromosome complement remaining in the embryonic soma after elimination.

The data presented below demonstrate that irradiation can influence the transmission of sex chromosomes through the male germ line and also through the female germ line, thereby giving rise to exceptional sex types.

MATERIAL AND RESULTS

Some of the data were obtained during the course of study of x-ray-induced sex-linked recessive lethals. Females heterozygous for the sex-linked markers Wavy (X′) and swollen (X) were bred to irradiated wild type males. Wavy and swollen are not alleles but they show practically no crossing-over (see Crouse, 1943). Among the progeny there occurred a number of exceptional sons in addition to the expected daughters. The data are presented in Table I. Thus, at dosages of 2000 r, 3000 r, and 4000 r exceptional sons were induced at the frequencies of 3.9%, 9.1%, and 7.2%, respectively. Moreover, all the exceptional males were matroclinous (swollen) for their sex-linked genes; none was patroclinous. These data are interpreted as follows: as a result of the irradiation, one sex chromosome instead of two was transmitted to the zygote through the sperm; then, following the elimination of this paternal X from the embryonic soma—as directed by the mother's genotype (XX′)—only one sex chromosome (maternal) remained and the embryo differentiated into a male.

TABLE I

Exceptional sons derived from W(sw) females bred to irradiated wild type males

Experiment	Dose	No. F_1 cultures	No. F_1 females	No. exc. + males	No. exc. sw males	% exc. sw males
M3X2	2000 r	9	337	0	14	$\frac{14}{351} = 3.9$
M3X3	3000 r	17	327	0	33	$\frac{33}{360} = 9.1$
M3X4	4000 r	22	593	0	46	$\frac{46}{639} = 7.2$

To establish with greater certainty that the exceptional males recorded in Table I arose not through x-ray-induced errors in chromosome elimination during embryogeny but rather through irregular sex chromosome transmission through the sperm, wild type males were x-rayed at 4000 r and bred to male-producing females. The following results were obtained: twelve F_1 cultures yielded a total of 234 sons and no exceptional daughters. Thus, the irradiation did not alter the kind of chromosome elimination determined by the XX constitution of the mother.

Another x-ray experiment was performed, this time on females, with a view to determining whether irradiation could induce irregular sex chromosome transmission through the female germ line. Newly-emerged female-producing females (oöcytes in first meiotic prophase) heterozygous for Wavy (XX′) were irradiated at 4000 r and bred singly to swollen males. Normally such matings should yield families of daughters. Instead, the progeny derived from 26 matings included 991 females, 18 exceptional swollen (patroclinous) males, zero exceptional wild type (matroclinous) males. Clearly, nondisjunction of sex chromosomes was induced by the irradiation; the patroclinous sons were derived from embryos which received no sex chromosome from the egg and which underwent the type of somatic chromosome elimination called for by the mother's genotype (XX′); thereby they became XO in constitution and consequently differentiated into males. It is interesting to note that differentiation into a male can proceed normally in Sciara under the influence of a paternal X chromosome.

Discussion

The data presented here support the view argued previously (Crouse, 1960), that the exceptional sex types which arise in unisexual families are not the result of errors in chromosome elimination in the embryonic soma; instead they arise from irregular sex chromosome transmission through the male or through the female germ line. Sex of the individual fly is controlled by the somatic complement which obtains following elimination. This point of view, of course, does not explain the condition observed in some species of Sciara where both bisexual and unisexual families occur regularly, and where individual strains exhibiting one or the other condition are interfertile (see Metz, 1938).

Summary

Among the progeny of female-producers bred to irradiated males there appear an appreciable number of exceptional sons, all of which are matroclinous for their sex-linked genes. Among the progeny of male-producers bred to irradiated males, no exceptional daughters occur. Finally, when female-producers are irradiated and bred to stock males, exceptional sons appear in the progeny and all of them are patroclinous for their sex-linked genes. These observations, taken collectively, are interpreted as follows: irradiation of sperm or of oöcytes can result in the transmission to the zygote of an irregular number of sex chromosomes; if, following elimination, a viable somatic complement (XX or XO) obtains, the embryo will differentiate accordingly into female or male.

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THE RESPIRATORY EXCHANGE OF DIAPAUSING PUPAE OF THE CECROPIA SILKWORM IN THE PRESENCE OF CARBON^{14} MONOXIDE[1]

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The inhibition of cytochrome oxidase by carbon monoxide stems from the remarkable similarity of this gas to oxygen. Recent work has shown that this similarity is even more far-reaching than formerly suspected, and that the combination of carbon monoxide with cytochrome oxidase may lead to phenomena in addition to a simple inhibition of respiration. For example, carbon monoxide may actually stimulate respiration (Daly, 1954). It may be combusted by oxygen, either enzymatically (Breckenridge, 1953), or catalytically; it may combine with water or alkali to produce formic acid or formates (Warburg, 1927).

The combustion of carbon monoxide has been studied by several workers including Clark, Stannard and Fenn (1950) and Black and Tyler (1959a, 1959b). The last-mentioned workers present a useful summary of the recent work on the oxidation of carbon monoxide, while the larger subject of the effects of carbon monoxide on living organisms has been ably reviewed by Lilienthal (1950).

Since the combustion of carbon monoxide may be superimposed on either the stimulation or the inhibition of respiration by carbon monoxide, the combustion of this gas has significant ramifications in studies of the carbon monoxide-inhibition of respiration. With present techniques for the measurement of radioactivity and for the analysis of small samples of respiratory gases, it is now feasible to disentangle the combustion of carbon monoxide from its effects on respiration.

The study reported here has combined these techniques to re-examine the respiratory exchange of diapausing pupae of the Cecropia silkworm in the presence of carbon monoxide and oxygen. A complete analysis has been achieved; namely, the simultaneous measurement of oxygen uptake, total gas uptake, and the production of $C^{14}O_2$ from $C^{14}O$. The results make possible a clear understanding of the complicated effects of carbon monoxide on the metabolism of diapausing pupae; they also provide new insight into the reaction of cytochrome oxidase with oxygen.

MATERIALS AND METHODS

1. *Experimental animals*

The experiments were performed on diapausing pupae of the Cecropia silkworm which were reared under nets on wild cherry trees and managed as described

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by Shappirio and Williams (1957). Certain pupae were used during the five-month period of stable diapause (Williams, 1956); others were placed in permanent diapause by the removal of their brains (Williams, 1946).

In all experiments using radioactive carbon monoxide, the most anterior abdominal spiracles were cannulated with a short section of 0.011-inch bore polyethylene tubing (Clay-Adams) to eliminate the intermittent release of carbon dioxide described by Schneiderman and Williams (1955) and Buck and Keister (1955). Since the cannulation sometimes resulted in minor integumentary injury, the pupae were routinely stored at 25° C. for at least a month prior to use.

During the CO-combustion experiments the pupae were maintained in the dark with occasional exposure to dim light. Since the cuticle of the pupae is quite opaque the experiments may be viewed as having been conducted in the dark.

2. *Manometric measurements of gas uptake*

The rate of uptake of oxygen (Q_{O_2}) by pupae in air at 25° C. was determined by a modification of Warburg's "direct method" (Schneiderman and Williams, 1953). For the purpose of absorbing carbon dioxide, two filter papers moistened with a total of 1.6 ml. of 1 N potassium hydroxide were sealed in each vessel. The Q_{O_2} was expressed as the microliters of oxygen consumed per gram of initial live weight per hour. The initial live weight is the weight of the pupa within two months after pupation.

The measurements of carbon monoxide uptake reported in Section 2 of the Results and of total gas uptake in CO-O_2 mixtures at atmospheric pressure, reported in Sections 1 and 8 of the Results, were performed by the technique just described.

3. *Volumetric measurements of total gas uptake*

a) *At elevated pressures*

Measurements of total gas uptake at elevated pressures were performed at 25° C. in 45-ml. capillary volumeters by the use of the high pressure technique of Schneiderman and Feder (1954).

b) *At atmospheric pressure*

Except for the results reported in Sections 1 and 8, all measurements of total gas uptake in CO-O_2 mixtures were performed at 25° C. in simple 8- or 15-ml. capillary volumeters (Fenn, 1935) similar to the one described by Schneiderman and Feder (1954) but modified as shown in Figure 1 to permit the sampling of gases. The volumeter was assembled and flushed with 20 volumes of the experimental gas introduced through the vaccine stopper by means of a 22-gauge needle, and expelled *via* the capillary and attached rubber tubing. The experimental gas was trapped in the volumeter at atmospheric pressure by withdrawing the hypodermic needle and clamping off the rubber tubing. Finally, the index drop was tipped into the capillary and brought to rest at the desired point by removing the clamp on the rubber tubing and adjusting the gas volume with a 5-ml. syringe inserted through the vaccine stopper and filled with the experimental gas mixture.

In each experiment one volumeter served as a thermobarometer. The latter was assembled exactly as the animal-containing volumeters except that a glass "space-occupier," displacing 6.7 ml., was substituted for the pupa.

In addition to the thermobarometer, one to four animal vessels were fastened to a Lucite holder and placed in a constant temperature bath at 25.0° C. and allowed to equilibrate for one-half hour. Barium hydroxide was then injected into the sidearm *via* the vaccine stopper. Further details of procedure are included in Section 3 of the Results.

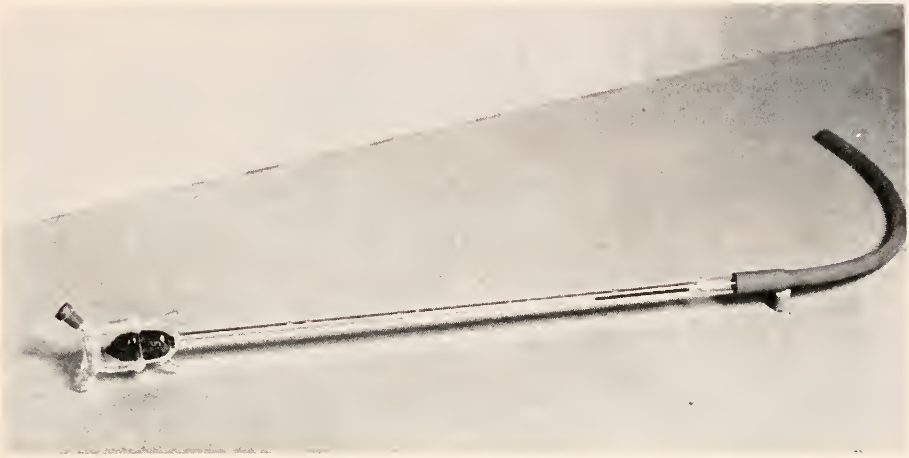

FIGURE 1. Volumeter used in the concomitant measurement at atmospheric pressure of total gas uptake, the oxygen uptake, and the production of carbon dioxide from carbon monoxide.

4. *Measurement of oxygen uptake in CO-O_2 mixtures by gas analysis*

Gas samples (ca. 1 ml.) were taken from each volumeter at the beginning and end of each experiment. The samples were analyzed for carbon dioxide and oxygen [3] in the Scholander half-cubic centimeter gas analyzer (Scholander, 1947), and the Q_{O_2} was calculated. The error introduced by the presence of carbon monoxide in the gas mixture was not in excess of 0.07% atm., as determined by analyses of carbon monoxide which had been purified as described below.

5. *Sampling and measurement of radioactive materials*

The $C^{14}O$ gas (New England Nuclear Corp.) was transferred by the method of Barton and Parrish (1956) as modified by Smetana (personal communication). Both $C^{14}O$-O_2 mixtures and $C^{14}O_2$ generated from $BaC^{14}O_3$ were counted in the gaseous phase in a Bernstein-Ballentine counting tube (Bernstein and Ballentine, 1950). The reproducibility in counting separate samples from the same gas mixture was in excess of 95%.

In certain experiments $BaC^{14}O_3$ was plated out on a millipore filter and counted as a solid on an automatic proportional flow counter (Baird Atomic; Model 750

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[3] The gas analyses were performed by Mr. John Steen.

PF). When the radioactivity was less than 100 counts per minute, solid samples were counted on a windowless Geiger flow counter (Laboratory Associates, Inc.).

The volume of CO_2 that came from CO was calculated from the relationship:

$$\text{volume}_{CO_2} = \frac{\text{dpm}_{CO_2}}{\text{dpm}_{CO}} \times \text{volume}_{CO} = \frac{k\ \text{cpm}_{CO_2}}{k'\ \text{cpm}_{CO}} \times \text{volume}_{CO}$$

Provided both $C^{14}O$ and $C^{14}O_2$ are counted under identical conditions, the constants k and k′ will be equal and the volume of carbon monoxide combusted can be calculated directly.

6. *Experimental gases*

The carrier carbon monoxide was the Matheson product (C.P.) assaying 99.9% carbon monoxide. This gas was passed through three serially arranged wash bottles, each equipped with a sintered glass dispersion element. The first bottle contained 10% potassium hydroxide to remove any residual carbon dioxide and carbonyl compounds; the second contained a saturated solution of lead acetate to remove any sulfur compounds; and the third vessel contained distilled water to saturate the gas with water vapor.

U.S.P. grade oxygen and U.S.P. grade carbon dioxide, each assaying 99.5%, were obtained from New England Gas Products, Inc. Since the major impurities in these gases were nitrogen, argon, oxygen (0.08% in the CO_2), and H_2O, no further purification was made.

Gas mixtures were prepared at atmospheric pressure and room temperature in a 6-liter, water-sealed spirometer. The gas composition was determined accurately with the Scholander gas analyzer (Scholander, 1947) immediately before use.

EXPERIMENTAL RESULTS

1. *Manometric evidence for the combustion of carbon monoxide*

In manometric experiments there is no quantitative way to distinguish between gas disappearance due to the combustion of carbon monoxide and that due to respiration. However, it is relatively easy to discover in a qualitative sense whether the combustion of carbon monoxide is going on. It will be recalled that this combustion involves the disappearance of one molecule of oxygen for every two molecules of carbon monoxide that are burned. A known volume of oxygen and an excess of carbon monoxide can be sealed in a Warburg flask, together with an animal and alkali. If all of the oxygen is removed by carbon monoxide-combustion, the amount of gas disappearing from the flask will be three times the known amount of oxygen present; if all of the oxygen is removed by respiration, the amount of gas disappearing will be equal to the amount of oxygen present; and finally, if part of the oxygen is removed by carbon monoxide-combustion and part by respiration, the total amount of gas disappearing will assume an intermediate value (Thimann *et al.*, 1954).

Six preparations of this type were assembled. In each preparation a diapausing pupa was sealed in a 45-ml. vessel together with a filter paper moistened with 1.6 ml. of 1 N potassium hydroxide. The vessels were each flushed with a slowly flowing mixture of 99% atm. carbon monoxide and 1% atm. oxygen for 15 hours and then sealed. The total amount of oxygen initially present in each animal vessel was therefore 1% of the gas volume of the sealed system. A seventh air-filled vessel served as thermobarometer.

The time course of total gas uptake in a typical preparation, corrected for the reaction of carbon monoxide with alkali as described below, is plotted in Figure 2. The amount of oxygen present at the outset is indicated on the graph. It will

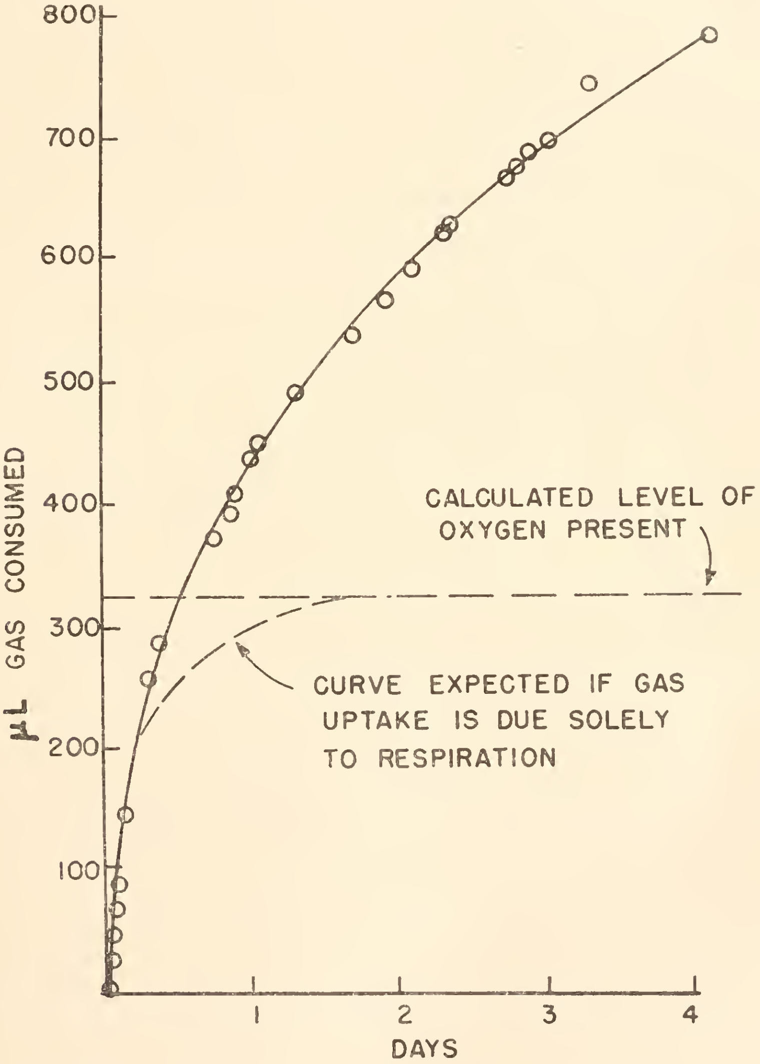

FIGURE 2. The time-course of the disappearance of gases from a sealed Warburg vessel containing a diapausing pupa in the presence of alkali and a gas mixture of 99% atm. carbon monoxide and 1% atm. oxygen. Each datum has been corrected for the reaction of carbon monoxide with the alkali.

be observed that gas continued to disappear long after the calculated 325 µl. of oxygen would have been used up. The substantial anaerobic capacity of the pupae (Harvey and Williams, 1958a; 1958b) enabled the continuation of the experiment for four days. At that time the function was tending toward a plateau somewhat short of the 975 µl. (325 µl. of oxygen plus 650 µl. of carbon monoxide) predicted for removal of the oxygen solely by carbon monoxide-oxidation. These results, together with the similar results obtained with the other five preparations, clearly demonstrate that carbon monoxide is removed from the system by the living pupa. Since this does not occur in the absence of oxygen (see Section 2) one may infer that carbon monoxide is being combusted.

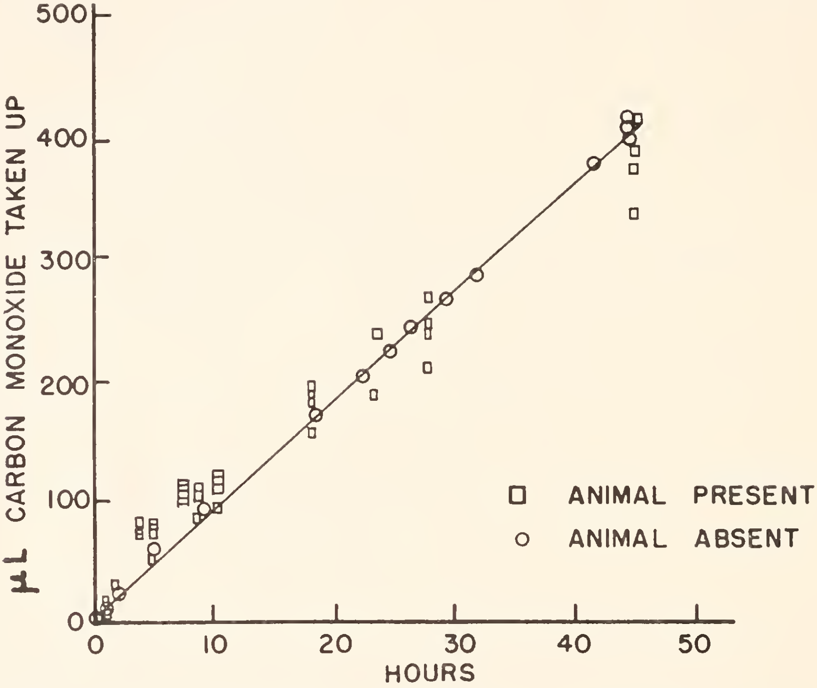

FIGURE 3. The time-course of the disappearance of carbon monoxide from Warburg vessels containing alkali and carbon monoxide, but no oxygen.

2. *Correction for the non-oxidative removal of carbon monoxide*

The manometric data just described were corrected for the reaction of carbon monoxide with alkali (Warburg, 1927) in the following way. Four 45-ml. Warburg vessels were prepared as in the above experiment but no animals were added. Three were flushed with 20 volumes of carbon monoxide that had been washed through potassium hydroxide and lead acetate (Method 6). The fourth vessel contained air and served as a thermobarometer. The uptake of carbon monoxide from each vessel, plotted as a function of time in Figure 3, amounted to 9.2 ± 0.2 (S.D.) µl./hour.

An essentially similar experiment was performed to determine whether the living pupa catalyzed any further non-oxidative uptake of carbon monoxide. When a pupa was added to the system the rate of carbon monoxide uptake was identical with that in the absence of the pupa (Fig. 3). Therefore, in the oxygen-free system there is no indication that the pupa catalyzes any further non-oxidative uptake of carbon monoxide.

The empirical correction factor for the reaction of carbon monoxide with alkali was subtracted from the gas disappearance in the experiment reported in Section 1. The absence of non-oxidative uptake of carbon monoxide by the diapausing pupa enabled an alternative method of correction in subsequent experiments. The thermobarometer was simply flushed with the experimental gas mixture . . . the thermobarometer correction then automatically compensated for the reaction of carbon monoxide with alkali.

In summary, we can state that the rate of gas disappearance in CO-O_2 mixtures is composed of a component due to the reaction of carbon monoxide with alkali, a component due to the combustion of carbon monoxide, and a component attributable to the respiration of the pupa. The experiment reported in the following section was designed to disentangle these latter two phenomena.

3. *Special technique for the concomitant determination of total gas uptake, oxygen uptake and production of carbon dioxide from carbon monoxide*

The total gas uptake ($\underset{\sim}{Q}_{\text{total}}$) of a pupa is equal to the oxygen uptake due to respiration ($\underset{\sim}{Q}_{O_2}R$), plus the oxygen uptake due to CO-combustion ($\underset{\sim}{Q}_{O_2}C$), plus the carbon monoxide uptake due to CO-combustion ($\underset{\sim}{Q}_{CO}$). The latter value is numerically equal to the CO_2 production due to CO combustion ($\underset{\sim}{Q}_{CO_2}C$).

$$\underset{\sim}{Q}_{\text{total}} = \underset{\sim}{Q}_{O_2}R + \underset{\sim}{Q}_{O_2}C + \underset{\sim}{Q}_{CO_2}C$$

From this relationship it is immediately clear that measurements of the total gas uptake and the oxygen uptake due to respiration and combustion combined allow us to calculate the amount of carbon dioxide production due to CO-combustion. In addition, this latter value can be determined directly using carbon^{14}-labeled carbon monoxide.

Each of four diapausing pupae was placed in a modified Warburg vessel and its oxygen uptake in air measured manometrically (Method 2). The pupae were then removed carefully from the Warburg vessels and each was sealed in a modified Fenn volumeter with a gas phase of 82% atm. CO and 18% atm. O_2, as described under Method 3b. In order to introduce a known amount of barium hydroxide, and of $C^{14}O$, and to remove samples for analysis of respiratory gases, the following techniques were employed in elaboration of the operations described in Method 3b.

The index drop of each volumeter was brought to the zero mark by withdrawing gas, using a hypodermic syringe equipped with a #25 blunted needle and lubricated with silicone grease. Then exactly 1.00 ml. of barium hydroxide (0.097 *M* $Ba(OH)_2 \cdot 8\ H_2O$) was added to the sidearm from a second 1-ml. syringe. Using a third 1-ml. syringe, approximately 1 ml of gas was removed for gas analysis, leaving the meniscus of the index drop at zero. Now 0.800 ml. of the same CO-O_2 mixture was added to each animal vessel and 0.500 ml. to the thermobarometer. Finally 0.200 ml. of $C^{14}O$ (ca. 36 μc./ml.) was added to each vessel so that the meniscus on each animal-containing volumeter now read 1.000 ml. and that on the thermobarometer read 0.700 ml. The excursions of the index drops were read at intervals of from 1 to 5 hours for a total period of 16 hours. During this same period three 0.200-ml. samples of gas were withdrawn from each volumeter to measure the specific activity of the $C^{14}O$ in the gas phase. As the pupa consumed gas the index drop moved toward zero. Pure oxygen was injected at convenient intervals to return the index drop to 1.000 and to restore the gas phase to its initial composition.

Finally, at the end of the experiment the index drops were all brought to zero by withdrawing gas; a second 1.000 ml. of barium hydroxide was added and the vessels shaken thoroughly to remove the last traces of unabsorbed CO_2. A final 1-ml. sample of gas was taken for analysis of respiratory gases. The vessels were removed from the bath and opened. The $BaCO_3$ was plated out on a millipore filter, oven-dried, and transferred to the gas-generating chamber on the manifold; the CO_2 was generated with concentrated H_2SO_4, introduced into the counting chamber and counted.

TABLE I

Comparison in normal diapausing pupae of the rate of oxygen uptake in air with the total gas uptake, oxygen uptake, and $C^{14}O_2$ production in a mixture of CO and O_2*

(a) Animal #	(b) In air Q_{O_2}**	In CO-O_2 mixture*			(f) Per cent of total uptake in CO-O_2 that is attributable to CO uptake
		(c) Q_{total}**	(d) Q_{O_2}**	(e) $Q_{C^{14}O_2}$**	
1085	13	22.7	22.0	1.38	6.1
1086	15	27.1	23.1	1.10	4.1
1087	11	23.7	21.6	1.57	6.6
1088	11	23.1	22.2	0.91	3.9
Average	12	24.1	22.2	1.24	5.2

* The gas mixture was 82% atm. CO and 18% atm. O_2.
** Q values in μl. per gram initial live weight per hour.

The rates of uptake of total gas, oxygen, and carbon^{14} dioxide are recorded for each of four animals in Table I, together with the corresponding rate of oxygen uptake in air. These data are representative of the results obtained with 48 pupae in 12 experiments essentially similar to the one being described.

4. *The stimulation of respiration by carbon monoxide*

Attention is first directed to columns b, c, and d of Table I. The average Q_{O_2} *in air* was 12 μl./gram/hour (column b). In the presence *of the CO-O_2* mixture the Q_{total} was almost exactly twice this value (column c). However, the Q_{O_2} *in this mixture,* as determined by direct gas analysis (column d), was nearly as high as the Q_{total} in this mixture. Therefore, the stimulation of gas uptake in the presence of CO and O_2 is due almost entirely to an increase in the rate of the true respiration of the pupa. (It should be emphasized that the term "respiration" refers to the normal metabolic process and does not include the uptake of carbon monoxide and oxygen associated with carbon monoxide-combustion.)

5. *The combustion of carbon monoxide to carbon dioxide*

Columns c and d of Table I show that in the case of each pupa the oxygen uptake in the CO-O_2 mixture is not quite as large as the total gas uptake. On the average the total gas uptake is about 2 µl./gram/hour greater than the average oxygen uptake. This discrepancy, together with the data reported in Section 1, suggests that carbon monoxide is being oxidatively removed from the vessels.

TABLE II

Calculation of total CO_2 that came from CO based on isotopic measurements

(a) Animal #	(b) *cpm per 0.200 ml. sample of original gas mixture**	(c) *cpm per 0.200 ml. of CO (calculated)	(d) *Total cpm in expired CO_2	(e) µl. of CO_2 that came from CO
Thermobarometer	112,000	92,000	−31	−0.068
1085	250,000	205,000	157,195	154
1086	147,000	121,000	54,324	101
1087	174,000	143,000	105,145	154
1088	53,400	43,800	19,466	89

* The values recorded have all been corrected for background activity (*ca* 500 cpm).
** The gas mixture was 82% atm. CO and 18% atm. O_2.

This conclusion is proven beyond reasonable doubt by the results recorded in column e. It is clear that $C^{14}O_2$ is being produced from $C^{14}O$ during the experiment at a rate of about 1.2 ± 10% µl./gram/hour. This amount of gas, together with the oxygen uptake (from gas analysis), accounts completely for the total gas uptake in the CO-O_2 mixture.

These data are presented in further detail in Table II. The background level of radioactivity in the $BaCO_3$ from the thermobarometer (column d) assures us that we are not dealing with a non-specific trapping of $C^{14}O$ in the alkali. Further isotopic experiments (Harvey and Smetana, unpublished observations) demonstrated that neither the catalytic effect of water on CO-combustion nor the solubility of $C^{14}O$ in the tissues of the pupae contributed in any way to the results reported in this paper.

6. *The fate of C^{14}-labeled compounds within the pupa*

a) *Under aerobic conditions*

At the end of the experiment described in Sections 3, 4 and 5, two pupae (nos. 1086 and 1087) were immediately desiccated under high vacuum for half a day. The gases that came from the pupae during this treatment were trapped in $Ba(OH)_2$. The $BaCO_3$ was treated with acid and the generated CO_2 was counted. The $C^{14}O_2$ given off in this way was added to that given off during the experiment and contributes to the $Q_{C^{14}O_2}$ reported in column e of Table I.

The desiccated remains of the pupa were ground up and oven-dried. Two

TABLE III

The fate of carbon^{14}-labeled compounds formed within the pupa during exposure to $C^{14}O$ under aerobic and anaerobic conditions

Animal	Condition during experiment	$C^{14}O_2$ (µl.) given off during experiment	$C^{14}O_2$ (µl.) distilled from pupa after experiment	C^{14}* compounds retained by pupa after distillation	
				Non-acidified residue	Acidified residue
1086	aerobic	80	10	11	5.6
1087	aerobic	127	21	5.9	2.0
1094	anaerobic	7.4	0.14	<0.4	<0.4
1095	anaerobic	11.1	0.13	<0.4	<0.4

* Expressed as µl. of $C^{14}O$.

aliquots were taken—one was counted directly and the other was acidified with 6 N HCl, washed with water, re-dried and counted in the same counter. Expressed as µl. of $C^{14}O$, there was an average of 8 µl. trapped in the pupa as calculated from the untreated aliquot, and an average of 4 µl. fixed as calculated from the acidified aliquot (Table III). Thus the reactions of CO and CO_2 combined could have resulted at most in the fixation of 4 µl. of $C^{14}O$.

b) *Under anaerobic conditions*

It is possible that the fixation of $C^{14}O$ is masked by the immediate oxidation of the resulting carbonyl compound by some aerobic process. To favor the accumulation of carbonyl compounds the following procedure was employed.

Each of two cannulated, brainless diapausing pupae was sealed in a volumeter containing $Ba(OH)_2$ with a gas phase of pure CO that had been passed through Fieser's solution (Fieser, 1924) (to assure the complete absence of oxygen from the gas phase), lead acetate, and water. The pupae were allowed to equilibrate for seven hours so that they might use up the last traces of oxygen dissolved within their tissues (Harvey and Williams, 1958a). Then $C^{14}O$ was added to give a final specific activity of 1 million cpm/ml. After 18 further hours each pupa was removed and its gases distilled into $Ba(OH)_2$ as described in the preceding section. The residue of each pupa was dried and divided into an acidified and an un-acidified fraction, each of which was plated out and counted. Each of the four aliquots yielded the normal background count (for this counter) of 18 to 19 cpm. Since this method is sensitive enough to detect the fixation of 0.4 µl. of gas, it is clear that no more than this amount of $C^{14}O$ was fixed under anaerobic conditions (Table III).

c) *The discharge of $C^{14}O_2$ from pupae*

When the experiment described in Sections 3, 4 and 5 was terminated, each of two pupae (nos. 1085 and 1088) was placed in a sealed container with $Ba(OH)_2$. The $BaCO_3$ was collected during each of three intervals from each pupa. The CO_2 was generated with acid and counted. The rate of discharge of $C^{14}O_2$ during and after the experiment is plotted as a function of the time after the experiment in Figure 4. It is clear that the rate of release of $C^{14}O_2$ declines rapidly and after 4 days almost all of the labeled gas has been expired. This $C^{14}O_2$ given off after the experiment was added to that collected during the experiment in the calculations of $Q_{C^{14}O_2}$ recorded for these pupae in Table I.

7. CO-combustion and the level of metabolism

It is possible to vary the level of metabolism in a diapausing pupa merely by rupturing the integument. Such injured pupae consume oxygen at rates several times that of uninjured individuals (Schneiderman and Williams, 1953). This

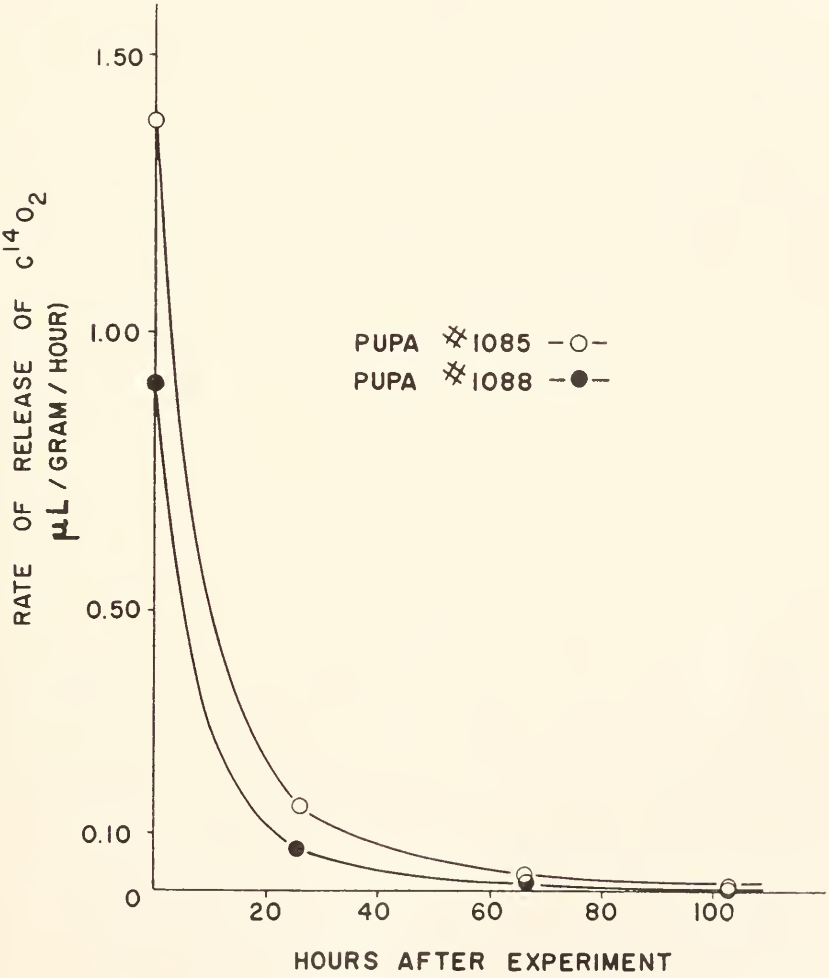

FIGURE 4. The rate of release of $C^{14}O_2$ from diapausing pupae, during exposure to a $C^{14}O$-O_2 mixture (plotted at 0 hours), and during the subsequent period after the exposure was terminated. It is seen that the rate of $C^{14}O_2$ expiration follows a decay function, and that by four days after the termination of exposure all of the radioactive gas has been expired.

TABLE IV

The rate of oxygen uptake of injured pupae in air compared with their total gas uptake and $C^{14}O_2$ production in a mixture of CO and O_2

Animal	In air Q_{O_2}**	In CO-O_2 mixture*		Per cent of total gas uptake that is attributable to CO-combustion
		Q_{total}**	$Q_{C^{14}O_2}$**	
943	76	91	3.22	3.5
944	26	37	1.12	3.0
963	36	38	1.41	3.7
Average	46	55	1.92	3.5

* The gas mixture was 82% atm. CO and 18% atm. O_2.
** Q values in μl. per gram initial live weight per hour.

phenomenon allows one to inquire into the relationship between metabolic rate and CO-combustion.

For this experiment each of three pupae was injured by the establishment of a terminal abdominal window (Williams, 1952). After a week the rate of oxygen uptake was measured in air, using the modified Warburg technique.

The pupae were then exposed to 82% atm. CO and 18% atm. O_2 exactly as described in the experiment in Section 3, except that no gas samples were withdrawn for gas analysis. The $C^{14}O_2$ given off during the experiment and during the 82 hours immediately following the experiment was plated out, generated with acid, and counted. In Table IV the Q_{O_2} in air, and the Q_{total} and the $Q_{C^{14}O_2}$ in the CO-O_2 mixture are presented.

It is clear from these somewhat preliminary data that carbon monoxide is combusted at a slightly higher rate in the injured pupa than in the uninjured pupa. There is some indication that there may be a linear increase in $Q_{C^{14}O_2}$ as a function of Q_{O_2} in air.

8. *The inhibition of the injury metabolism by carbon monoxide*

Having dealt quantitatively with the combustion of carbon monoxide in both normal and injured diapausing pupae, we are in a position to evaluate the effects of this gas on the true respiration of the pupa.

The rate of oxygen consumption of each of 24 brainless diapausing pupae was first measured in air (Method 2). The individuals were then divided into three groups and treated as follows. The rate of gas disappearance of each of nine pupae was re-determined in a CO-O_2 mixture of 90% atm. carbon monoxide and 10% atm. oxygen ($CO/O_2 = 9/1$); the rate for each of nine pupae in 93% atm. carbon monoxide and 7% atm. oxygen ($CO/O_2 = 13/1$); and (making use of the high pressure technique) the rate for each of six pupae, in 500% atm. carbon monoxide and 21% atm. oxygen ($CO/O_2 = 24/1$). The results in each case were corrected for the reaction of carbon monoxide with alkali but not for the combustion of CO to CO_2 (see Discussion (3) for correction for CO-combustion).

Similar determinations were made on a total of ten brainless diapausing pupae which, one week previously, had been subjected to a large leg injury (Schneiderman and Williams, 1953).

The results are summarized in Figure 5 along with data from the pupae exposed to a mixture of 82% atm. carbon monoxide and 18% atm. oxygen ($CO/O_2 = 5/1$) described in Sections 3 and 5 of the Results. (For simplicity, the results in the $CO\text{-}O_2$ mixture of 13/1 are not presented in the Figure.)

The ratio between the rates of decrease in gas volume in $CO\text{-}O_2$ mixtures and in air have been computed for each animal and plotted as a function of the corre-

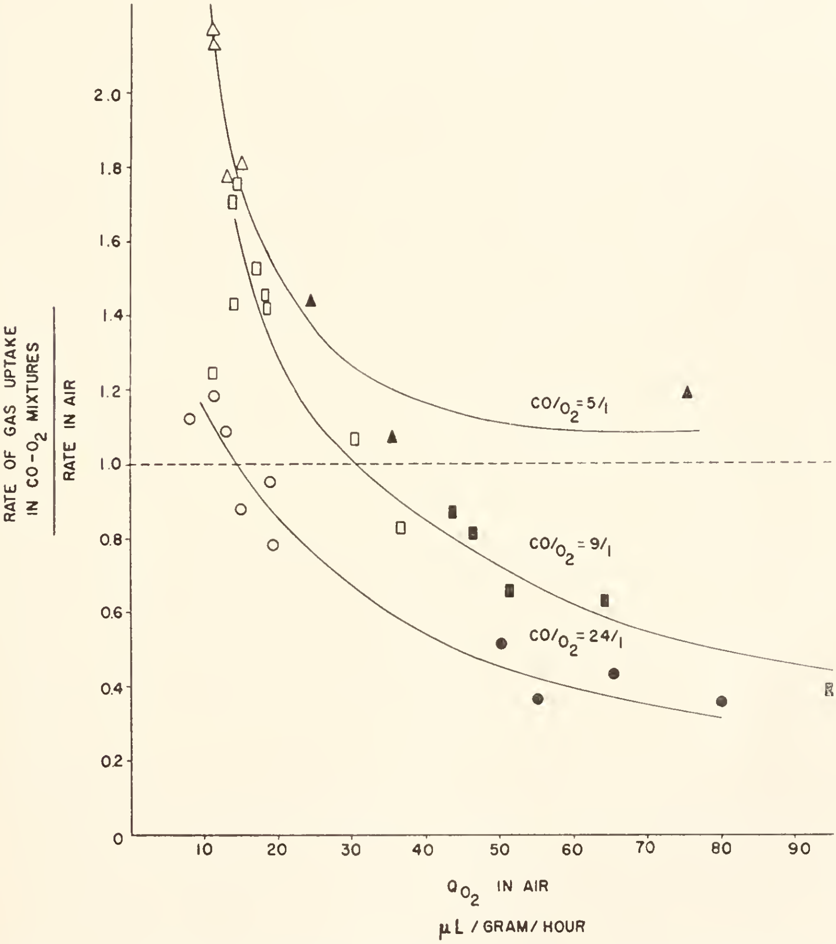

FIGURE 5. For a series of uninjured and injured pupae of the Cecropia silkworm, the relative rates of gas uptake in carbon monoxide *versus* air are plotted as a function of the Q_{O_2} of the corresponding animals in air. Each datum has been corrected for the reaction of carbon monoxide with alkali but not for carbon monoxide-combustion. All points above the dotted line signal an acceleration of gas uptake by carbon monoxide. Points below this line signal a depression by carbon monoxide. The open points are uninjured pupae; the solid points are injured pupae.

sponding Q_{O_2} in air. It will be observed that for animals exposed to the mixture of 82% atm. CO and 18% atm. O_2 this ratio is greater than 1, no matter what the prior Q_{O_2} in air. This means that the gas uptake is invariably higher in the presence of this CO-O_2 mixture than it is in air. When the amount of carbon monoxide in the mixture is increased (lower curves) it will be noted that this ratio remains greater than 1 only for pupae exhibiting a low Q_{O_2} in air. In pupae whose metabolism has been enhanced by injury, the ratio is invariably less than 1, indicating that the rate of gas uptake is inhibited. Finally in each of the lower curves there is an intermediate level of metabolism where carbon monoxide neither stimulates nor inhibits.

Discussion

1. *Carbon monoxide-combustion and cytochrome oxidase*

Most of the evidence in the literature suggests that the combustion of carbon monoxide by oxygen is mediated by cytochrome oxidase (Breckenridge, 1953; Black and Tyler, 1959b). The most damaging evidence for this hypothesis is the augmentation of CO-combustion in light (Stannard, 1940; Black and Tyler, 1959a) where, presumably, the cytochrome oxidase-carbon monoxide complex is very unstable. However, Black and Tyler (1959a) attempt with some success to account for this light-stimulation of CO-oxidation. Stannard (1940) and Chance (1949) have implied that the combustion of carbon monoxide is mediated by catalase. However, Breckenridge (1953) exposed several purified enzyme preparations, including catalase, cytochrome *c* and diaphorase, to mixtures of 80% atm. $C^{14}O$ and 20% atm. O_2. He showed that only cytochrome oxidase was able to cause the formation of $C^{14}O_2$; moreover, this activity was augmented as the enzyme was purified.

As a third alternative, Wald (1959) has proposed that an intermediate (RH) might be carbonylated to the corresponding aldehyde (RCOH). The latter might subsequently reduce water, yielding two hydrogens, while the aldehyde was being oxidized to the acid (RCOOH). The hydrogens would be available as substrate for the cytochrome system while the acid could be decarboxylated to yield carbon dioxide and thereby regenerate the intermediate (RH) which would thus act as a catalyst.

The results of the present study argue against the formation of a carbonylated intermediate. The failure to detect carbon^{14}-labeled material in the dried residue of the anaerobic pupa (Table III) argues that no direct fixation of $C^{14}O$ occurred. The remote possibility exists, however, that the small amount of $C^{14}O_2$ given off during the anaerobic experiment may have been the oxidation product of some carbonylated intermediate. Only a trace of carbon^{14}-labeled compound other than $C^{14}O_2$ was found in the aerobic experiment where as many as 127 μl. of this gas were formed (Table III). This small aerobic carbon^{14} fixation was undoubtedly due to the fixation of some endogenous $C^{14}O_2$ produced within the pupa. Finally, it will be recalled that there was no manometric evidence for carbon monoxide uptake by the anaerobic pupa (Fig. 3).

These findings fail to support Wald's theory of the carbonylated intermediate. Neither do they entirely disprove it, since the fixation of less than 0.4 μl. (9 μ

moles) of carbon monoxide could not be detected by this procedure. Possibly an accumulation of less than 9 μ moles of intermediate would be sufficient for Wald's carbonylation-catalytic process.

The simplest interpretation of these data is that both $C^{14}O$ and O_2 react directly with cytochrome oxidase to form $C^{14}O_2$. Some of the $C^{14}O_2$ is exhaled immediately as a gas, some goes into solution in the tissues, some exchanges with tissue bicarbonate, eventually to be given off as a gas, while presumably some of the $C^{14}O_2$ is fixed (Table III).

This interpretation would seem to require either that CO and O_2 react in sequence with cytochrome oxidase or, more simply, that they react simultaneously with this enzyme. This second prospect implies that there is more than one combining site on the cytochrome oxidase molecule. Wald and Allen (1957) find that, in the reaction of carbon monoxide with cytochrome oxidase, the coefficient n is greater than 1 in the Hill equation. This finding implies an interaction at the catalytic site between more than one molecule of carbon monoxide on cytochrome oxidase which, in turn, implies the presence of more than 1 heme per active molecule.

Ambe and Venkataraman (1959) report that in highly purified, homogeneous preparations of cytochrome oxidase there is but one heme per monomer. However, they also find one copper atom per heme group. Of the greatest importance was their finding that their monomer displayed no cytochrome oxidase activity unless phospholipide was added.

These seemingly contradictory observations are reconciled by the hypothesis that several individual units (monomers) of cytochrome oxidase are oriented on the phospholipide in such a way that interaction between two or more monomers is possible.

Although there are many other interpretations of these data, the combustion of carbon monoxide by molecular oxygen is a fact which must be accounted for, and an instrument which must not be overlooked, in efforts to understand the mechanism by which cytochrome oxidase activates oxygen.

2. *Carbon monoxide and the metabolism of diapausing pupae*

The diapausing pupa is particularly responsive to carbon monoxide. In no other animal known to us has CO-stimulation of respiration been clearly demonstrated.

CO-combustion, on the other hand, is well-known in a variety of animals. In Cecropia pupae this reaction accounts for but from 5 to 7% of the total gas uptake in CO-O_2 mixtures, in marked contrast to the 57% found in frog muscle (Clark, Stannard and Fenn, 1950). In developing sea urchin eggs the values range from 10 to 43% (Black and Tyler, 1959a). It is tempting to speculate that all aerobic cells are capable of catalyzing CO-oxidation and that the magnitude of the phenomenon varies from animal to animal depending perhaps, as Breckenridge (1953) has suggested, on the state of oxidation of their cytochrome oxidase.

3. *Carbon monoxide-inhibition of the true respiration of injured pupae*

When the gas uptake due to CO-combustion is subtracted from the total gas uptake, the true respiration in CO-O_2 mixtures is revealed. From Tables I and

IV we see that (with a CO-O_2 ratio of 5 to 1) we must subtract 1.8 μl./gram/hour for CO-combustion of normal diapausing pupae and 2.9 μl./gram/hour for injured pupae. Extensive semi-quantitative experiments (Harvey, unpublished) show that at higher CO-O_2 ratios the gas uptake due to CO-combustion is somewhat less, though still easily detectable with radioactive material. Since the correction is small compared to the total gas uptake of injured pupae, the values mentioned above are sufficiently accurate for our present purposes.

Thus the effects of CO-O_2 mixtures on the true respiration of pupae follow the same functions as those presented in Figure 5 except that each curve is shifted for from 0.2 to 0.4 units toward the abscissa. This means that the stimulation of true respiration by carbon monoxide is somewhat less than that indicated by considerations of the total gas uptake, and the inhibition of true respiration is somewhat greater.

This CO-inhibition of the true respiration of injured pupae is of considerable theoretical interest. Harvey (1956) reports that the total gas uptake of injured pupae is inhibited by carbon monoxide—a finding subsequently confirmed by Kurland and Schneiderman (1959). We can now state that the true respiration of injured pupae is inhibited to an even greater extent. Harvey and Shappirio (unpublished observations) have shown that this augmented CO-sensitivity of the injury-enhanced true respiration of pupae is attributable to an increase in the activity of certain cytochrome enzymes.

I would like to express my appreciation to Professor Carroll M. Williams for his critical reading of the manuscript, and to Mr. Frank Smetana and Mr. John Steen for their technical advice and assistance.

SUMMARY

1. After due correction for complicating reactions, the rate of total gas uptake of normal diapausing pupae of the Cecropia silkworm in a carbon monoxide-oxygen mixture of 5 to 1 is found to be stimulated approximately 2-fold over the rate in air.

2. Ninety per cent of this *extra* gas uptake is due to a stimulation of the true respiration of the pupa.

3. The remaining ten per cent is due to the combustion of carbon monoxide to carbon dioxide.

4. The combustion of carbon monoxide is increased slightly when the metabolism of the pupa is enhanced following integumentary injury.

5. There is a slight fixation of carbon-14 in pupae under aerobic conditions, presumably as a result of fixation of $C^{14}O_2$ manufactured from $C^{14}O$ by the pupa.

6. There is no detectable fixation of $C^{14}O$ in pupae exposed to this gas under anaerobic conditions.

7. After correction for carbon monoxide-combustion, it is clear that the enhanced oxygen uptake following integumentary injury of diapausing pupae is inhibited by carbon monoxide.

8. This carbon monoxide-inhibition is directly proportional to the enhancement of oxygen uptake following injury.

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EXPERIMENTS ON THE NORMAL MEIOTIC BLOCK IN THE OVUM OF TRITURUS VIRIDESCENS[1]

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At the time of penetration of the amphibian egg by the sperm, the second division of meiosis is arrested at metaphase. Entrance of the sperm triggers the activation of the blocked division and the second polar body is produced. Much interest has, through the years, been centered about the problem of just what triggers the completion of the division as a part of the "activation" of the egg. Equally fascinating, but largely ignored, is the question of the nature and origin of the meiotic block which is removed by the entrance of the sperm.

The variation in the stage of meiosis customarily reached by the eggs of the major animal groups before fertilization has been known for many years (for references see Wilson, 1925; Just, 1939; Dalcq, 1957; Rothschild, 1956). In the vertebrates, the egg is usually fertilized at metaphase of the second maturation division, and it is commonly stated that the division in unfertilized eggs is blocked at this stage. This statement holds for most amphibian eggs, although variations have been noted for *Siredon* (*Ambystoma*) (Schultze, 1887) and for *Triturus viridescens* (Humphries, 1955, 1956). As will be shown in this paper, the exceptions in *T. viridescens* seem to occur only under certain circumstances.

In earlier work (Humphries, 1956) it was shown that in *Triturus viridescens* the first maturation spindle forms at about the time of ovulation, and the first polar body is extruded soon after the egg enters the oviduct. The second division figure then forms and remains at metaphase until fertilization occurs. Coelomic eggs which, for any reason, are delayed in entering the oviduct, extrude the first polar body in the coelom and show the formation of the second metaphase figure in the same way as do oviducal eggs. However, two of the coelomic eggs in that study had passed the usual stage of arrest and were in anaphase II. It seemed possible that the difference between the oviducal and coelomic environments might be responsible for the escape from the block in these eggs and, further, that the oviducal environment might be a factor in the normal blocking of meiosis in unfertilized eggs. The present report deals with an investigation of these possibilities, using two approaches: (1) ligation of the oviducts at several levels, to retain the eggs abnormally long in the coelom or in certain regions of the oviducts, and (2) maintenance of eggs *in vitro*, with and without jelly coats.

MATERIALS AND METHODS

Ligation experiments: Adult *Triturus viridescens* females were obtained from ponds near Charlottesville, Virginia, and Franklin and Canton, North Carolina,

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and kept in a refrigerator at about 12° C. until used. The oviducts in this species show a gross differentiation into distinct regions (Adams, 1940) which have been identified, from anterior to posterior, as regions A through E (Humphries and Hughes, 1959). An ostial portion, which is apparently non-secretory, precedes region A. The deposition of jelly about the egg begins as the egg passes through region A. The oviducts were ligated as close to the ostium as possible, or at the posterior limits of the A, C, or D regions. Following ligation, the females were allowed to recover for a period of a few days to two weeks, after which they received a subcutaneous implantation of one or two intact anterior lobes of the pituitary of *Rana pipiens*. If two lobes were used, the second implantation followed the first by 48 hours. Animals were sacrificed and opened 48 or 72 hours after the final pituitary implantation and any eggs which had accumulated in the coelom or oviducts were removed, placed in Bouin's fixative, embedded in Tissuemat, sectioned at 10 microns, and stained with Harris' haemalum and fast green. In the first experiments done, using only animals with oviducts ligated at the ostium, four advances past metaphase II were obtained from a total of 66 eggs which had reached at least metaphase II. Two were from "48-hour animals," and two were from "72-hour animals." It apeared, therefore, that 48 hours was an adequate time interval in which to expect advances and this was used for all remaining ligation experiments. The eggs used in these experiments were obtained from a total of 43 animals. Statistical analysis was made through the use of the contingency chi-square (Mather, 1943), using the Yates correction.

In vitro experiments: Animals used in this part of the study were obtained from lakes near Franklin and Highlands, North Carolina. In this group ovulation was stimulated by the injection into the coelom of 0.25 to 0.40 cc. of chorionic gonadotropin (Antuitrin "S," Parke, Davis, 500 units per cc.). When necessary, a second injection was given, usually 48 hours after the first. Eggs present in the coelom, ostium, and secreting parts of the oviducts were removed following decapitation of the animals, and were placed in solutions in covered syracuse dishes, the bottoms of which, in most instances, had been covered with paraffin to prevent adhesion of eggs to the glass. In some cases, the bottom was covered with filter paper wet with one of the solutions used. About a third of the oviducal eggs were freed mechanically from their jelly, using watchmaker's forceps, before being placed in solution. The solutions employed were Holtfreter's solution, Ringer's solution, and Niu-Twitty solution. Some eggs were kept in small droplets of solution, barely sufficient to cover them, while others were kept in well filled dishes. In the case of eggs kept in droplets, and in the case of those kept on wet filter paper, the covers of the dishes were sealed with Vaseline. Most of the eggs were kept at 20° C. in a water bath for 24 hours, though some were kept for 12 or 48 hours. Fixation and processing were the same as outlined for the previous experiments, except for a few eggs which were stained in toluidine blue for cytochemical study. The eggs used in this part of the study were taken from a total of 49 animals.

The statistical method used was the same as for the results of the ligation experiments.

OBSERVATIONS

Ligation experiments: The stages of meiosis in eggs of the various categories are given in Table I. Ovulation occurs over an extended period in this species;

thus, some of the eggs were probably out of the ovary and in the coelom or oviducts for two days or more prior to fixation, while others may have been released from the ovary only a few minutes before the animal was sacrificed. There is no way of knowing, other than in a general way, based on position in the oviduct and stage of meiosis, just how long a given egg has been out of the ovary. Under the circumstances, one would expect to find the entire spectrum of stages of meiosis, ranging from metaphase I through metaphase II and perhaps beyond. Such a situation is reflected in the distribution shown in Table I.

TABLE I

Stages of meiosis in oocytes removed from coelom and oviducts of operated animals 48 or 72 hours following final pituitary implantation*

Source	Earlier than Metaphase II	Metaphase II	Anaphase II to Telophase II	Meiosis complete
Coelom—ovid. not ligated at ostium	43	81	0	0
Coelom—ovid. ligated at ostium	32	44	0	1
Ostium—ovid. ligated in ostial region	17	35	1	3
Region A—ovid. ligated post. end of "A"	7	14	0	0
Region A—ovid. ligated post. end of "C"	1	10	0	0
Region A—ovid. ligated post. end of "D"	1	0	0	0
Region B—ovid. ligated post. end of "C"	1	0	0	0
Region C—ovid. ligated post. end of "C"	3	167 (51 had no jelly)	0	2**
Region D—ovid. ligated post. end of "C"	1	3	0	0
Region D—ovid. ligated post. end of "D"	2	38	0	0
Coelom—escaped from oviduct—jelly-covered	1	58	0	0
Totals	109	450	1	6

* See text.
** Not jelly-covered.

Meiosis was judged to be complete under one of the following conditions: presence of two polar bodies; presence of two polar bodies plus a single set of single chromosomes (monads) within the egg (Fig. 3); presence of a set of monads within the egg, in the absence of one or both polar bodies. In these eggs the single chromosomes within the egg were never in other than the condensed condition of mitotic chromosomes, nor were they surrounded by a nuclear membrane. In the one case of anaphase II which was observed, single chromosomes were about half-way to the poles and the first polar body was present (Figs. 1 and 2).

Meiosis was found to be advanced or complete only in eggs with no jelly coats. Advances occurred in coelomic eggs, in eggs from the ostial (non-jelly-secreting) region of the oviducts, and in non-jelly-covered eggs from region C of the oviducts. Region C is a secreting region, and eggs in this region ordinarily have jelly coats deposited by the C region and by more anterior parts of the oviduct, but in a few instances the ligated oviducts became so distended with eggs that eggs which were

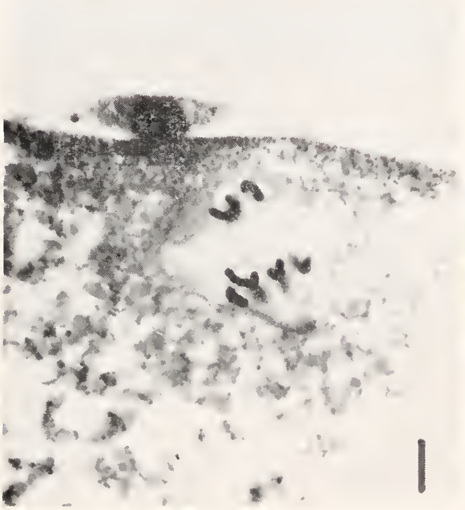
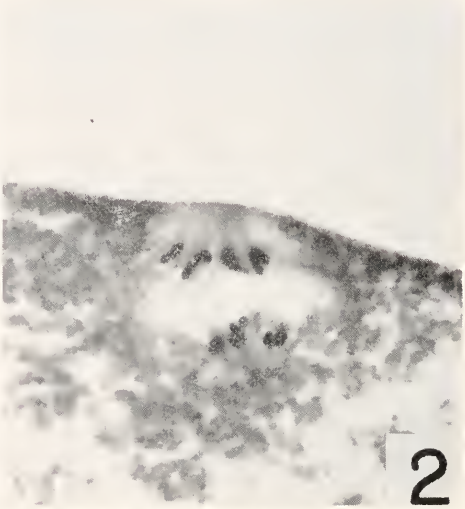
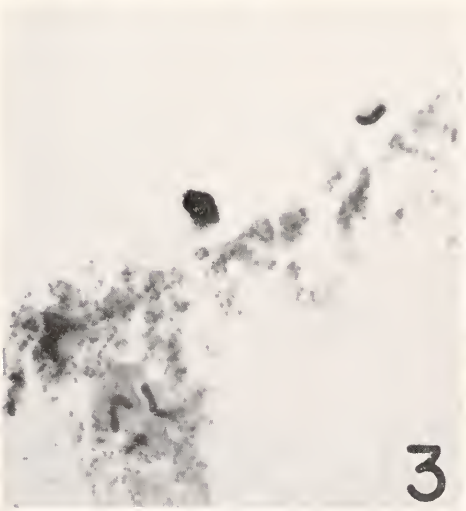
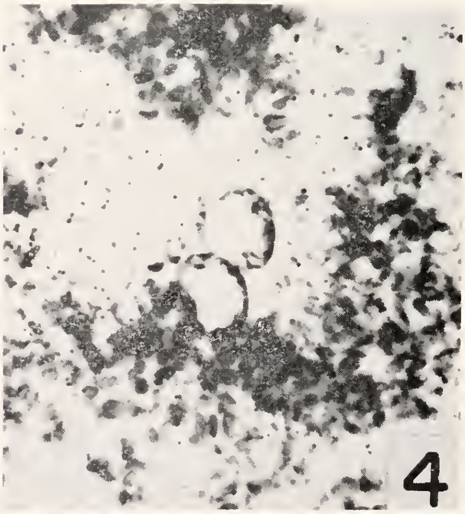

Figure 1. Anaphase II with adjacent polar body I, from the ostium of an oviduct ligated as close as possible to the anterior end. × 790.

Figure 2. Section adjacent to that shown in Figure 1. × 790.

Figure 3. Completed meiosis in an egg from the coelom of an animal with oviducts ligated at the ostia. Three chromosomes of the haploid set of eleven can be seen at the lower left. Portions of polar bodies I and II are visible outside the egg. × 970.

Figure 4. Two interphase nuclei in an egg from the coelom kept for 40 hours in Niu-Twitty solution. × 770.

TABLE II

Comparison of number of advances past Metaphase II in eggs with no jelly coats and eggs having jelly coats in ligation experiments

	Metaphase II	Later than Metaphase II	Total
With jelly	229	0	229
Without jelly	130	7	137
Totals	359	7	366

centrally located within the mass apparently never came in contact with the secretory epithelium and obtained no jelly. The eggs from region C in which advances were observed were of this type.

The frequency of advance past metaphase II in jelly-covered and non-jelly-covered eggs is given in Table II. Eggs in stages earlier than metaphase II and eggs which were probably not actually delayed by a ligature (*e.g.,* coelomic eggs taken from an animal whose oviducts were ligated toward the middle or posterior part) are not included. Statistical analysis of the distribution gives $P = < .01$.

In vitro experiments: Information as to the stages found in the 254 eggs of the four types kept *in vitro* is given in Table III. No advances past metaphase II occurred in jelly-covered eggs, and only one doubtful advance occurred in the eggs from which the jelly had been removed. Advances were found in 22% of the eggs never exposed to jelly. Comparison of the frequency of advance in eggs exposed to jelly (including those from which the jelly was removed) with those never exposed to it gives $P = < .001$.

TABLE III

Stages in eggs maintained in vitro (24 hours except in cases noted)

Type of egg	First meiotic division	Metaphase II	Anaphase II or Telophase II	Meiosis complete, condensed chromosomes or none found	Pronucleus or pronuclei	Post-meiotic "mitosis"	Total
Coelomic	0	73 (three for 12 hrs. only)	6 (one for 12 hrs. only)	1	2 (one for 12 hrs., one for 40 hrs.)	11	93
Ostial	0	8	2	1	0	0	11
Oviducal, with jelly	0	103 (four for 12 hrs., eleven for 48 hrs.)	0	0	0	0	103
Oviducal, jelly removed	0	46 (one for 12 hrs., eight for 48 hrs.)	1?	0	0	0	47
Totals		230	9	2	2	11	254

The solution in which the eggs were kept seemed to have no effect on the frequency of advance nor on the "normality" of the divisions, although eggs kept in Ringer's solution could usually be distinguished microscopically from those kept in Holtfreter's or Niu-Twitty solution by their "more dense" appearance. Also, the amount of fluid surrounding the egg seemed to be of little, if any, importance.

In eleven of the twenty coelomic eggs in which advance occurred, post-meiotic mitotic activity was occurring. Such "mitoses" often occurred well beneath the egg surface, as in a cleavage division, in contrast to meiotic divisions, which usually occur at the surface. In several instances it appeared that a haploid set of monads was being randomly distributed to the poles, since, even at anaphase, the total number of chromosomes on the spindle was only the haploid number. There were no asters nor was there any evidence of attempted cytoplasmic division. Such "mitosis" probably accounts for the "pronuclei" which were seen in one egg (Fig. 4).

DISCUSSION

There are two general alternatives which may be suggested as possible explanations of the source of meiotic arrest in the amphibian egg. One is that the source is within the egg itself, the other is that the block is somehow imposed upon the egg through some aspect of the environment. The former seems to be the explanation in eggs of *Habrobracon,* where von Borstel (1957) has found evidence that blockage is under genetic control. A third possibility is that a combination of internal and external factors is involved. Not nearly enough is known of either the physiology of the amphibian oocyte or of its environment to allow a ready choice between the alternatives.

Probably the explanation most attractive at present is that proposing a blocking mechanism intrinsic to the egg. The results of the present study should be examined from this point of view. If this is done, certainly one feature of the data seems to agree strongly with the hypothesis of an intrinsic blocking mechanism, and that is the fact that the great majority of eggs which had progressed as far as the second division had not proceeded beyond metaphase of that division. This was true even of non-jelly-covered eggs, the only type in which the block was evaded. The implication is that the process normally stops at this stage, and that, rather infrequently, it is capable of proceeding to completion. If nothing else, it appears that there is a lengthy pause at metaphase. However, in many cases there was little indication as to how long the divisions had been in progress, and undoubtedly some of the eggs had only recently reached metaphase II, and could not have been expected to have advanced beyond that stage. If intrinsic blockage is assumed, however, then what mechanism could account for the evasion of the block in the eggs of this study? It is possible that degenerative changes within the eggs somehow triggered the completion of the second division, but if this is the case, the jelly deposited by the oviducts must protect against such changes or the effects of such changes, since no jelly-covered egg showed advance. Also, eggs which are laid, but not fertilized, will degenerate in their jelly coats without advancing past the normal stage of blockage.

Under the assumption of intrinsic blockage, perhaps the most acceptable hypothesis for explaining the present results is that some aspect of the environment, either the natural coelomic or ostial environment or the artificial ones, was effective in stimulating completion of the division, perhaps through stimulation of the division apparatus directly or through a general stimulation of some aspect of the metabolism of the egg. Again, however, the jelly coats seem to protect, this time against a possible external stimulatory effect, since jelly-covered eggs which escaped from the oviducts back into the coelom, or which were kept *in vitro* for as long as 48 hours, were never more advanced than metaphase II.

The present results should also be viewed in the light of the possibility that normal meiotic blockage is a function of some factor of the environment of the egg, and not of the egg itself. Under this hypothesis, meiosis might be considered to be a process which, once having been initiated, would, in the absence of outside interference, continue to completion. If an outside inhibitor is considered, the oviducal secretions, specifically the jelly layers deposited about the oocyte, seem to be especially suspect as a source of inhibition. Previous workers (Bataillon and Tchou-Su, 1930; Dalcq *et al.*, 1936) have considered the oocytes of anuran amphibians to be "intoxicated" with carbon dioxide, presumably related to the secretions of the oviducts. Such an "intoxication" might itself be the cause of meiotic blockage, which would perhaps be thought of as an indirect effect of the oviducal jelly.

There is also the possibility of a more direct effect of the oviducal secretions. The first division is usually completed and the second division spindle is usually formed in the anterior part of the oviduct (Humphries, 1956). The division spindle is located with one pole at the egg surface, where it might be unusually sensitive to an outside effect. These facts make the idea of a possible effect of oviducal secretion worth consideration, especially in the light of the results of the present study. The lack of advance in jelly-covered eggs is itself important in this consideration, but the fact that only one doubtful case of escape from blockage occurred in the eggs from which the jelly was removed before their stay *in vitro* suggests that mere exposure to the jelly, not necessarily its presence, may somehow be effective in preventing advance. Recent studies on the histochemistry of the oviducts of urodeles (Kambara, 1956a, 1956b, 1957a, 1957b; Humphries and Hughes, 1959) have shown the presence of acid mucopolysaccharide in the anterior regions of the oviducts, while Minganti (1955) has reported a heparin-like anticoagulant activity of amphibian egg jelly. Heilbrunn and his co-workers, as well as others, have repeatedly shown that compounds of this type are capable of antimitotic activity and that they are of widespread occurrence (see especially Heilbrunn, 1956, and Heilbrunn *et al.*, 1957). Others, working with eggs of the sea urchin, have apparently implicated mucopolysaccharides in general inhibitory activity prior to fertilization (Runnström and Immers, 1956). Certainly the present findings require a serious consideration of the possibility of a direct or indirect effect of the oviducal secretions.

The exploration of the problem of meiotic blockage thus far does not allow a choice between the major alternative explanations. It is apparent that the jelly layers are of significance with regard to blockage under the conditions of these experiments, and they may be involved, as well, in blockage as it occurs under natural conditions. However, the jelly may be important only as an insulator from the environment. Were the jelly the source of the meiotic block, one would expect all eggs unexposed to jelly to complete meiosis if given ample time and suitable environment. If we assume that these conditions were met by the *in vitro* experiments reported here, in which most such eggs showed arrest, then it is not possible to maintain that the jelly is the sole factor in meiotic arrest, although it may play an important ancillary role, such as the maintenance of an already established block.

SUMMARY

1. The oviducts of the newt, *Triturus viridescens,* were ligated at several levels to retain oocytes from 48 to 72 hours in the coelom or in certain regions of the oviducts. Eggs were collected and studied to determine the stage of meiosis, with the finding that advances past metaphase II, the normal stage of arrest, occurred at a low frequency, but were confined to eggs never exposed to the jelly secreted by the oviducts.

2. Twenty-two per cent of the eggs removed from the coelom or ostium of the oviduct and kept *in vitro* advanced past metaphase II, but eggs with jelly coverings did not advance. One doubtful advance was observed in an egg from which the jelly had been removed prior to its stay *in vitro.* Some "advanced" eggs exhibited post-meiotic "mitotic" activity.

3. The general problem of meiotic blockage in the egg is discussed. The jelly secreted by the oviducts is of significance with regard to meiotic arrest under the conditions of the experiments reported, and may play some role in normal arrest. The evidence does not, however, allow a decision as to the source of meiotic inhibition.

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HUMIDITY REACTIONS OF SOME AQUATIC ISOPODS IN THE AIR

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In nearly all groups of terrestrial invertebrates the occurrence of behavioral reactions to the humidity of air has been established. In addition to the different groups of terrestrial arthropods mentioned, *e.g.,* by Lagerspetz and Jäynäs (1959), earthworms (Heimburger, 1924) seem to exhibit humidity reactions. In an amphibious vertebrate, *Triton,* behavioral reactions to the humidity of the air have also been found to occur (Czeloth, 1930).

For aquatic invertebrates, the present authors have been unable to find in the literature any data on their possible humidity reactions when transferred to air. Maxwell (1897) found that specimens of *Nereis,* when placed on the border of moist and dry sand, more often crept to the moist side. In this, as well as in some other cases, the animals apparently reacted to the contact stimuli, probably by contact chemoreception. In any case, the results of such experiments do not exclude the possibility of contact reception. The field experiments on the orientation of aquatic invertebrates when transferred to dry land, performed by Weiss (1914) and Turner (1924), among others, indicate that in all cases when the animals have returned to the water in numbers surpassing the chance expectation, this has been due to optical stimuli. Thus the reactions of aquatic invertebrates to the humidity of the air seem never to have been tested by methods commonly used in the study of humidity reactions in terrestrial animals. The evidence about the orientation of aquatic invertebrates towards water, in fact, strongly suggests the guidance by optical stimuli only, and not by humidity gradients in the air.

Those aquatic invertebrates which *a priori* could be thought to exhibit humidity reactions are those (1) which inhabit shallow pools which may dry up during warm, rainless periods, and (2) which are considered to be either ancestors or descendants of terrestrial forms. Terrestrial animals have been evolved from aquatic ancestors. As the capacity for behavioral regulation of the body water content has been found to occur in all terrestrial forms studied, it is possible that the capacity for humidity reactions already existed in their last aquatic ancestors. Especially as the first terrestrial animals were apparently less well adapted to encounter the low humidities often locally found in the atmosphere, the occurrence of humidity reactions in these pioneers of the dry land would have rendered them a great selective advantage. In addition, humidity reactions can only be found in those aquatic animals, (3) which are able to live for some time even when exposed to the air. This condition, as well as the ability to move even when subjected to terrestrial conditions, is, of course, necessary also for the demonstration of the humidity reactions by the current experimental methods.

The above conditions are satisfied by certain aquatic members of the crustacean groups Isopoda and Amphipoda. In both of these groups terrestrial forms have evolved separately from aquatic ancestors, and the morphological and physiological similarities between the recent aquatic and terrestrial species are greater than in most other groups with both aquatic and terrestrial species.

The test animal chosen was an aquatic isopod, *Asellus aquaticus*. It is an inhabitant of fresh water in the littoral zone of lakes and occurs in especially dense populations in shallow pools and ponds. It is also a common inhabitant of the *Fucus vesiculosus* zone in the brackish water of the Baltic and is found to occur there up to a salinity of 8‰ (records of *Asellus* exist even from 15‰). Phylogenetically, the Asellota are considered primitive. According to some observations, they are able to survive in the mud bottom of pools which have dried up (Franz, 1924, p. 467). Turner (1924) does not give any quantitive data, but states (p. 53) that "the movements of neither pond snails, asellids nor dragonfly larvae are influenced by nearby bodies of water."

For the sake of comparison, another aquatic isopod, *Idotea baltica,* was used in some experiments. This is a typical brackish-water animal, which occurs in numbers among the *Fucus* vegetation. All members of the Idoteidae are marine or brackish-water forms.

MATERIAL AND METHODS

The specimens of *Asellus aquaticus* were caught either from fresh-water pools in the parks of the city of Turku, SW Finland, or from brackish water (salinity about 6‰ in the vicinity of the Lohm Marine Biological Station, University of Turku) in the archipelago about 40 kilometers southeast from the city of Turku. From the latter locality, specimens of *Idotea baltica* were also collected. The animals were kept in their native water in polyethylene or glass containers at room temperature (19–21° C.), at which all experiments were also performed.

For the study of humidity reactions, the alternative chamber method of Gunn and Kennedy (1936), as modified by Perttunen (1953), was adopted. In order to obtain different relative humidities in the air of the two halves of the alternative chamber, dried silica gel with a moisture indicator was placed on one side and water on the other. The theoretical difference in the R.H.s between the sides would thus be 0–100%; the R.H.s measured by Edney paper hygrometers placed on the two sides on the false floor of brass wire gauze were about 40% and 99%, respectively. The procedure followed was otherwise that described by Perttunen (1953). The total number of position records was 2586.

In some experiments, single isopods were placed in the alternative chamber and their tracks and the periods of time spent in motion and on each side of the apparatus were recorded. The length of the different portions of the tracks were then measured with the aid of a map-measurer.

In order to find out the survival time in atmospheres with different R.H.s, the animals were kept in small glass vials in a desiccator over dried silica gel (R.H. 0%), or in a desiccator over a water surface. In the latter case, the inner surface of the desiccator was covered with wet filter paper strips extending to the water. Thus R.H. of 100% was obtained. No food or water was given to the animals. Ten animals of each group were weighed at intervals of one to eighteen hours. Animals which showed no spontaneous movements and failed to react to repeated tactile stimulation were regarded as dead. For some experiments, the antennae and antennulae were carefully removed with fine scissors.

RESULTS

1. *The humidity reactions in the air*

The results of the humidity reaction experiments performed with *Asellus aquaticus* both from fresh and brackish water, with antennectomized animals as well as with *Idotea baltica,* appear in Table I. Both the fresh- and brackish-water specimens of *Asellus* reacted to the difference between the humidities of the air by spending more of the time in the humid part of the chamber. This reaction was more pronounced in the brackish-water specimens. The removal of the antennae and antennulae did not alter the outcome of the experiments. Thus the receptors involved in the humidity perception in *Asellus* seem not to be located on the antennae or antennulae.

TABLE I

The humidity reactions of Asellus aquaticus *and* Idotea baltica

	Number of test animals	Distribution of position records	Chi square	p	Intensity of reaction
Asellus aquaticus					
Fresh-water specimens:					
Intact	150	W* 285 D* 165	32.0	<0.0005	+26.7%
Antennectomized	80	W 205 D 115	25.3	<0.0005	+28.1%
Brackish-water specimens:					
Intact	150	W 413 D 187	84.4	<0.0005	+37.5%
Antennectomized	154	W 408 D 208	64.3	<0.0005	+32.3%
Idotea baltica	150	W 315 D 285	1.5	<0.2	+ 5.0%

* W, position records on the moist side; D, position records on the dry side.

Further attempts to locate the humidity receptors were made by removal of one pair of thoracic appendages and by removal of the pleopods. These operations always resulted in disturbance of the movements of the animals and the removal of the pleopods finally caused their death. Thus, by this method, no positive results concerning the location of the humidity receptors could be obtained.

The other species studied, *Idotea baltica,* did not show any definite reaction to the humidity of the air. The distribution of the animals in the alternative chamber was not found to deviate significantly from the chance expectation.

2. *The orientation types involved in the humidity reaction*

Tracks followed by 10 specimens of *Asellus* during 10 minutes in the alternative chamber were analyzed. The time spent in the dry side of the chamber was an average of 1.8 minutes (variation from 0 to 4 minutes), while in the wet side of the chamber 8.2 minutes were spent as an average by the test animals (variation from 6 to 10 minutes). When approaching the boundary between the sides of the apparatus, the animals sometimes turned through an angle of 180° and then moved back to the side from which they had approached the boundary. This happened in 9 cases when the animal was coming to the boundary from the humid side, and never when the animal was coming to the boundary from the dry side of the chamber. Thus the klinotactic orientation mechanism (Fraenkel and Gunn, 1940) seemed to play a role in the humidity reactions of *Asellus.*

The mean velocities of the movement of the animals counted for each side of the chamber were the following: 18.1 cm. per minute in the dry and 7. 9 cm. per minute in the humid side. The mean number of stops per minute was on the dry side 1.2 and on the humid side 1.4. The differences between these values were not statistically significant. The mean number of random turnings per minute was 4.0 in the dry and 0.8 in the humid side of the apparatus. Owing to the large variation in the individual scores, even this difference was not statistically secured (p between 0.2 and 0.1). Although these results would suggest the unimportance of the orthokinetic and klinokinetic orientation mechanisms in the humidity reactions of *Asellus,* the tendency for orthokinesis and klinokinesis appearing from the results was especially strong in those individuals which failed to show klinotactic behavior.

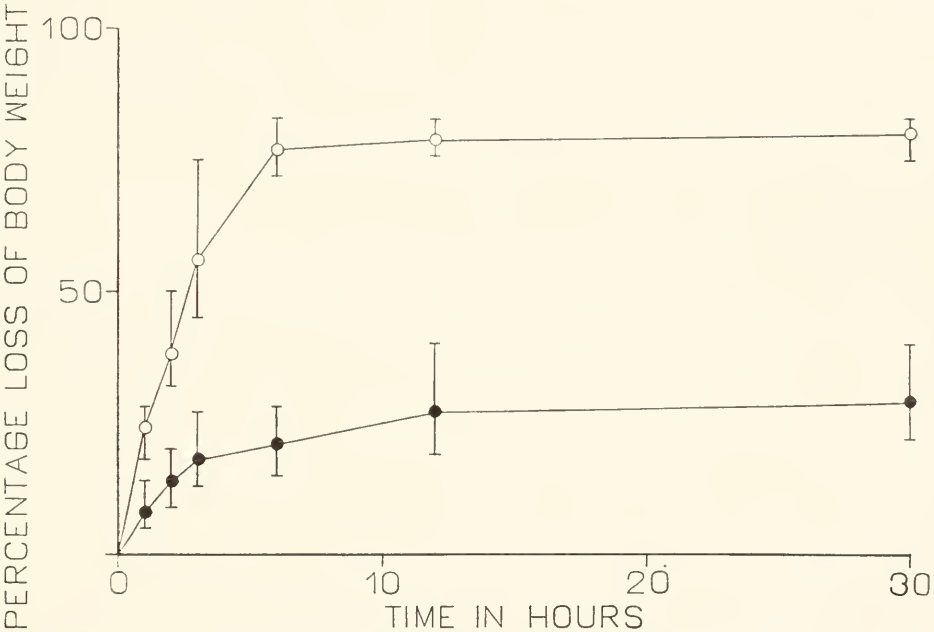

FIGURE 1. Percentage loss of body weight of *Asellus aquaticus* in dry atmosphere (upper graph) and in atmosphere saturated with water vapor (lower graph) at 20° C. Variation range is shown at each mean.

3. *The survival time and the rate of water loss in the air*

When specimens of *Asellus* (mean initial weight 22 mg.) were subjected to dry atmosphere in a desiccator containing dried silica gel, all animals in less than one hour became unable to regain their normal position if turned on their backs. In one hour the animals had lost an average of 24% of their body weight (Fig. 1). Fifty per cent of the animals died in less than two hours (mean weight loss 38%), and not one survived for longer than four hours. Of those animals (mean initial weight 22 mg.) subjected to a saturated atmosphere in a desiccator containing water, 50% lived for more than 30 hours (mean weight loss 20%), and one for more than three days. Thus greater humidity of the air definitely enhances the survival of *Asellus* when out of water, by decreasing the rate of water loss.

According to Palmén and Suomalainen (1945), the mean lethal weight loss during desiccation was 34 to 43% for the terrestrial isopod, *Porcellio scaber*, at 20° C. This was attained in 12 to 18 hours. The present results show that the lethal amount of water loss during desiccation was approximately the same in *Asellus aquaticus*. The water content of *Asellus* was about 80%.

DISCUSSION AND CONCLUSIONS

In addition to the amphibious vertebrate, *Triton* (Czeloth, 1930), an aquatic invertebrate, *Asellus aquaticus,* has now been shown to exhibit behavioral reactions to the humidity of the air. When exposed to air, the selecting of a humid habitat apparently greatly increases the possiblities of survival in this species by decreasing the water loss. Such behavioral reactions have certainly been advantageous for the first terrestrial and amphibious forms of isopods. The fact that the antennae or antennulae do not contain the receptors mediating the response to the humidity of the air, in addition to the fact that the salinity receptors of this species have been shown to be mainly located on the antennae (Lagerspetz and Mattila, 1961), seems to indicate that humidity perception is not a modified form of salinity perception in this animal, but a separate receptive capacity.

Further attempts to locate the humidity receptors were futile because of the trouble caused by the operation to the animals. Humidity reactions have been earlier found to occur in terrestrial isopods by Gunn (1937) and by Waloff (1941). However, Gunn (1937) failed to demonstrate any humidity receptors in wood-lice. Gunn suggested that the receptors probably lie in the thoracic region. Waloff (1941) also pointed out the possibility that the concentration of body fluids in a dry atmosphere would in itself have an effect on the reactions of the animals through proprioceptors. A detailed electrophysiological study of the external receptors and proprioceptors of isopods would probably be necessary in order to gather further information on this point.

The humidity orientation mechanisms of terrestrial isopods have been studied closely by Gunn (1937), who considered the orthokinetic orientation to be the most important orientation mechanism in the humidity reactions of isopods, and by Waloff (1941), who found both orthokinesis and klinokinesis to be of importance in the humidity orientation of terrestrial isopods. The present results suggest that also the klinotactic type of orientation is important for the selection of humid habitats by *Asellus,* when taken out of water.

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SALINITY REACTIONS OF SOME FRESH- AND BRACKISH-WATER CRUSTACEANS

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Knowledge of the behavioral reactions of aquatic invertebrates to chemical stimuli has been reviewed by Warden, Jenkins and Warner (1940) and, more recently, by Hodgson (1955). The reaction thresholds of various invertebrate animals have been determined for various substances, especially for those known to stimulate the vertebrate chemoreceptors, *e.g.*, for several salts and alcohols. In addition, the part played by chemoreception in food recognition has been amply documented. The methods of study have been based either on the use of alternative chambers and gradient apparatus or on the conditioning of the behavior of the animals. In addition, the physiology of the chemoreceptors in some aquatic invertebrates has recently been studied by electrophysiological means (*Callinectes,* Hodgson, Lettwin and Roeder, 1955; *Limulus,* Barber, 1956; *Cambarus,* Hodgson, 1958).

The major part of the Baltic has a low and stable salinity. However, along the coasts of the northern Baltic, there occur periods of extreme dilution of the surface water, owing to the melting of snow and ice and the increased fresh-water output of the rivers in the spring. In such conditions, the capacity of selecting appropriate salinities could have a survival value for the brackish-water animals.

The behavioral discrimination of various salinities has been demonstrated in three semi-terrestrial crustaceans, *Ligia baudiniana* (Barnes, 1935, 1938, 1940), *Birgus latro* (Gross, 1955), and *Pachygrapsus crassipes* (Gross, 1957). In addition, Krijgsman and Krijgsman (1954) have found the South-African rock lobster, *Jasus lalandii,* to be capable of osmoreception. Earlier observations of Giersberg (1926) on *Octopus vulgaris* indicate that the reaction thresholds for salinity are in this species too high to allow the behavioral reactions to occur in natural conditions. Spiegel (1927) found that *Crangon vulgaris* reacted to higher concentrations of sea water than those found in its natural habitat.

As only a few studies dealing with the reactions of aquatic invertebrates to differences in the concentration of natural sea water were available, the salinity reactions of some fresh- and brackish-water crustaceans were studied by using an alternative chamber suitable for aquatic animals. The apparatus of Hodgson (1951) was adopted as the experimental device.

The following problems were studied: (1) Do the animals behaviorally discriminate between waters of different salinities? (2) Which are the concentrations preferred? (3) Are the reaction thresholds low enough to allow the reactions to play any part in the natural orientation of the animals? Additional information was sought on the following points: (4) Are the reactions to pure

NaCl solutions similar to those to diluted natural sea water? (5) Where are the receptors mediating the salinity reactions placed?

As test animals, the following crustaceans were used: *Asellus aquaticus* (Isopoda) both from fresh and brackish water, *Idotea baltica* (Isopoda) from brackish water, and *Gammarus* spp. (Amphipoda) from brackish water. It appeared that of the specimens of *Gammarus* used in experiments, 88% belonged to the species *G. oceanicus*, 7% to *G. locusta*, 3% to *G. zaddachi*, and 2% to *G. salinus*.

Material and Methods

For the experiments with fresh-water *Asellus*, the animals were caught in two shallow ponds in the parks of the city of Turku, southwestern Finland, in October, 1958, and in May and October, 1959. The brackish-water specimens of *Asellus* were collected in June, 1959, together with specimens of *Idotea* and *Gammarus* from the *Fucus vesiculosus* vegetation at Lohm Marine Biological Station in the Finnish southwestern archipelago, about 40 km. southwest from Turku. The experiments with brackish-water animals were performed at the Lohm Station. In the laboratory, the test animals were kept in their native, aerated water in polyethylene containers at room temperature (19–21° C.).

The apparatus of Hodgson (1951) was only slightly modified, *i.e.*, the water flowing to the funnels D in Hodgson's Figure 1 came directly from two separate storage bottles containing the solutions in question. Care was taken to have the temperatures of the two solutions equal. In some preliminary experiments, water at different temperatures was allowed to flow through the sides of the reaction tube. When the difference between the temperatures was less than 5° C., the distribution of the animals in the apparatus did not deviate from the chance expectation. In the salinity reaction experiments, the temperature difference was always less than 0.1° C. The pH of the solutions was also controlled. Groups of 10 or 20 specimens were transferred to the alternative chamber through its left end, or, in every second experiment, through its right end. The number of animals on each side of the chamber was recorded at intervals of two minutes. At intervals of 10 minutes, the tubing screws of the apparatus were adjusted so that the solutions changed sides. The mean flow rate from the reaction tube to the sink was 200 ml. per minute. The duration of the experiment was 40 to 60 minutes. All experiments were performed at room temperature (19–21° C.). The control experiments with stained solutions showed that the solutions mixed only in a 0.5 cm. zone in the middle of the chamber. After the change of the sides, the distinct boundary was re-established in one minute. When one solution at the same temperature was allowed to flow through both halves of the chamber, the test animals showed no preference for either side. Except for specific purposes, the animals were used only once for the experiments. Altogether, 1290 test animals were used and their positions in the reaction tube were recorded 26,950 times.

Results

1. *Experiments with Asellus from fresh water*

From the results of the experimental series 1, presented in Table I, it appears that the test animals were distributed at random in the chamber when the alternatives were tap water and Baltic brackish water with a salinity of 5.4‰.

The reaction threshold for pure NaCl was more closely studied, using fresh-water *Asellus*. The results of these experiments (series 2–6) appear in Table I. There are several ways of defining the threshold values, the most common being the intensity of the stimulus which evokes a response 50% of the times it is applied. Thus, if the animals in question have, through chemoreception and appropriate orientation mechanisms, selected one of the solutions in 50% of the cases when they have approached the boundary of the solutions, and the other 50% of the animals are distributed at random between the two solutions, 75% of the animals should be on one side and 25% on the other side of the boundary, when the threshold value for the reaction is reached. Another way of measuring the capacity for discriminative behavior is to determine the upper limit for the zone of indifferent reaction (U.L.I.R.). This is the maximal value of the stimulus which does not evoke a statistically significant response. The reaction threshold of fresh-water *Asellus* for NaCl was about 10‰, while the U.L.I.R. for tap water and NaCl was below 1‰.

TABLE I

Salinity reactions of Asellus aquaticus *from fresh water*

Exp. series	Test solutions	Number of test animals	Distribution of position records	Chi square	*p*
1	Tap water 5.4‰ B.W.*	30	311 289	0.8	0.4
2	Tap water 15‰ NaCl	60	944 256	394	<0.0005
3	Tap water 10‰ NaCl	60	908 292	316	<0.0005
4	Tap water 5‰ NaCl	60	814 386	153	<0.0005
5	Tap water 2‰ NaCl	30	346 256	14.1	<0.0005
6	Tap water 1‰ NaCl	30	477 273	55.5	<0.0005
7	5‰ NaCl 15‰ NaCl	60	831 369	178	<0.0005
8	5‰ NaCl 10‰ NaCl	60	679 503	31.2	<0.0005
9	10‰ NaCl 15‰ NaCl	60	700 500	33.3	<0.0005

* B.W., brackish water.

The differential reaction threshold for pure NaCl was also studied with

TABLE II

Effect of antennectomy on the salinity reaction of Asellus aquaticus *from fresh water*

Exp. series	Test solutions	Number of test animals	Distribution of position records	Chi square	p
10	Tap water 15‰ NaCl	60 antennectomized	566 634	3.85	<0.05
11	Tap water 15‰ NaCl	60 antennectomized after 1–3 days	673 527	17.8	<0.0005

Asellus. The results of series 7–9 are given in Table I. The differential reaction threshold is defined as the difference of the stimulus values for which 50% of the animals show reaction, the others being distributed at random. The differential reaction threshold on both sides of 10‰ NaCl is more than 10‰ (*i.e.,* higher than from 5 to 15‰). In these, as in the previous cases, specimens of fresh-water *Asellus* preferred the lower concentration alternative.

The position of the receptors responsible for the reaction to NaCl solutions was studied by recording the reactions of antennectomized specimens of *Asellus.* Series 10 was performed immediately after the removal of the antennae and antennulae and series 11 one to three days later with the same animals (Table II). When compared with the data from series 2, presented in Table I, it appears that antennectomy destroyed the reaction to NaCl. Thus, the chemoreceptors mediating this reaction are apparently situated for the most part on the antennae and/or antennulae in *Asellus.*

2. *Experiments with Asellus from brackish water*

The results of the experiments with brackish-water specimens of *Asellus* are presented in Table III. The results of series 12 show that even the specimens

TABLE III

Salinity reactions of Asellus aquaticus *from brackish water*

Exp. series	Test solutions	Number of test animals	Distribution of position records	Chi square	p
12	Tap water 6‰ B.W.	60	585 615	0.75	0.4
13	Tap water 10‰ NaCl	60	820 380	161.4	<0.0005
14	Tap water 5‰ NaCl	60	705 495	36.7	<0.0005
15	Tap water 1‰ NaCl	60	592 608	0.2	0.7

of *Asellus* living in the brackish water do not discriminate between tap water and their native 6‰ brackish water.

In order to determine the reaction threshold for pure NaCl solutions in brackish-water *Asellus*, experimental series 13–15 were performed. It was found that the reaction threshold is above 10‰, the U.L.I.R. for tap water and NaCl being about 1‰.

3. *Experiments with Idotea baltica*

For the sake of comparison, the reactions of a typical brackish-water isopod, *Idotea baltica,* were tested in tap water *versus* its native 6‰ brackish water. Sixty test animals were used. The animals were recorded 476 times on the side with fresh water and 724 times on the side with brackish water, the difference being significant (chi square 51.2, $p < 0.0005$).

4. *Experiments with Gammarus spp.*

As the bulk (88%) of the test animals belonged to the species *Gammarus oceanicus,* the results given here will probably mostly reflect the chemoreceptory conditions in this species. The results are presented in Table IV. *Gammarus* was the only animal used in this study which strongly reacted to the difference between tap water and 6‰ brackish water (series 17). As its preference for the brackish water seemed to be pronounced, its reactions to smaller salinity differences were tested for brackish waters with salinities of 5 *versus* 6‰ (series 18). The result, however, did not differ significantly from the chance expectation.

TABLE IV

Salinity reactions of Gammarus *spp.*

Exp. series	Test solutions	Number of test animals	Distribution of position records	Chi square	p
17	Tap water 6‰ B.W.	60	109 1091	804	<0.0005
18	5‰ B.W. 6‰ B.W.	60	577 623	1.76	0.2
19	Tap water 6‰ B.W.	60 without antennulae	320 880	261	<0.0005
20	Tap water 6‰ B,W,	60 without antennae	216 984	492	<0.0005
21	Tap water 6‰ B.W.	60 without antennae and antennulae	598 602	0.01	>0.9
22	Tap water 6‰ NaCl	60	546 654	9.7	<0.01
23	Tap water 6‰ NaCl	60 without antennae and antennulae	597 603	0.03	>0.9

In order to find the position of the chemoreceptors involved in the salinity perception in these amphipods, the antennulae were removed from 60 animals and the reactions of the animals to the difference between tap water and 6‰ brackish water were tested (series 19). When compared with the results of series 17, it was found that the intensity of the reaction was reduced. The intensity of the reaction can be calculated by the following formula:

$$\frac{A - B}{N} \cdot 100 = R.$$

If A and B represent the numbers of records of animals on each side of the reaction tube and N is the total number of position records, then R is the excess percentage of records on the side with A animals. The intensities of reaction measured as the excess percentages on the side with 6‰ brackish water, calculated for the experimental series 17 and 19–21, were as follows:

17: 6‰ brackish water *vs.* tap water, normal animals	+81.8%
19: as above, antennulae removed	+46.7%
20: as above, antennae removed	+64.0%
21: as above, antennulae and antennae removed	+ 0.3%
Chance expectation	0.0%

Thus, the removal of both antennae and antennulae totally abolished the salinity reactions in *Gammarus*. The removal of antennulae, only, reduced the intensity of the salinity reaction, as did the removal of the antennae, although to a lesser extent. It seems safe to assume that *Gammarus* has the chemoreceptors responsible for orientation in a salinity gradient on its pairs of antennulae and antennae, the antennulae being apparently somewhat more important in salinity perception.

It seemed worthwhile to explore whether *Gammarus*, which preferred brackish water with a salinity of 6‰ (expressed as NaCl), would react similarly to pure 6‰ NaCl solutions. Thus, the experiments of series 22 (Table IV) were performed. The intensity of the reaction was much weaker than for 6‰ brackish water, but still positive for the saline medium (excess percentage + 9.0%). The removal of antennulae and antennae (series 23) also in this case abolished the salinity preference reaction, the excess percentage being reduced to 0.5%. The receptors involved in the chemoreception of pure NaCl are thus also situated on the antennulae and/or antennae.

DISCUSSION

1. *The significance of salinity reactions in nature*

It was shown that the specimens of *Asellus* from fresh and from brackish water did not behaviorally react to the difference between tap water and Baltic brackish water with a salinity of 5.4‰ or 6‰. These salinities are approximately those which usually prevail in the natural habitats of *Asellus aquaticus* in wide areas of the northern Baltic. As no reaction occurs for this large difference, the compensation for the much smaller salinity variations (occurring, for example, on the shores as a consequence of the melting of the ice in spring) by movement of the animals to other habitats seems utterly improbable.

Specimens of *Gammarus* clearly reacted to the difference between fresh water and 6‰ brackish water, but they did not show a significant response to the difference between 5‰ and 6‰ brackish water. The reaction of *Idotea baltica* to the difference between fresh water and 6‰ brackish water was even less pronounced than that of *Gammarus*. Thus, the adaptive value of behavioral reactions to differences in salinity in the crustaceans studied does not seem to have been established by the results of this study. However, the methods of investigation may be criticized for not allowing longer times than 10 minutes for the discriminative reactions to the alternatives studied. However, in several cases with pure NaCl solutions, this period of time was found to be entirely sufficient for the performance of definite responses by most of the animals. The possible significance of behavioral salinity reaction mechanisms in other truly aquatic brackish-water animals, for example in estuarine forms, deserves further attention.

2. *The salinity reaction thresholds*

Most of the experimental series were devoted to the determination of salinity reaction thresholds. It appeared that the reaction threshold for NaCl is somewhat higher for the brackish-water specimens of *Asellus* than for the fresh-water ones. Similarly, the U.L.I.R. between fresh water and NaCl is higher in the brackish-water specimens. It would be interesting to find out whether this is a result of a sensory or some other physiological adaptation process, or an indication of physiological race formation. As reported previously (Lagerspetz, 1958), and afterwards confirmed by repeated tests, the brackish-water specimens of *Asellus* do not survive a longer stay in fresh water, while the fresh-water specimens are apparently not injured by a transfer to Baltic brackish water. Thus, one could at least study the effects of the stay of the fresh-water specimens in brackish water on their salinity reaction threshold.

The experiments for the determination of the differential salinity reaction threshold in *Asellus* showed that these animals are not only capable of discriminating fresh water from saline solutions, but also react differently to different concentrations of NaCl.

Fresh water or the weaker NaCl solution was preferred by *Asellus,* which showed no significant reaction to the brackish water. However, the brackish-water specimens were rather more often found on the side with brackish water, and the fresh-water animals slightly more often on the side with fresh water. In all cases, the number of animals on the brackish-water side was significantly higher for brackish-water animals. *Gammarus* slightly but significantly preferred 6‰ NaCl to fresh water. Such a comparatively strong NaCl solution was very definitely rejected by fresh-water *Asellus,* but also to a lesser degree by the brackish-water specimens. The reaction of *Gammarus* in brackish-water *versus* fresh-water experiments was again strongly positive towards brackish water. Thus, the following modifications may be linked with the progressive adaptation of fresh-water animals to brackish water:

(1) The rise of the NaCl reaction threshold by the development of a slight preference for dilute solutions of NaCl to fresh water.

(2) The development of a strong preference for brackish water to fresh water.

3. *The location of chemoreceptors*

The location of chemoreceptors in Crustacea has been documented and discussed in some twenty papers since 1887. Most experiments hitherto made have been performed on Decapoda. Among the exceptions are the early observations of May (1887) on *Mysis flexuosa* (Mysidacea), studies by Abraham and Wolsky (1930) on land isopods, by Uchida (1930) on the sex recognition in *Asellus,* and by Seifert (1930) on *Triops cancriformis* (Branchiopoda).

The chemoreceptors may be specialized to mediate responses only for certain chemical substances. Thus, the different receptor types may also have different sites in the animal. The results of experiments with one or a few substances cannot thus be generally applied to the receptors for other substances. It is therefore understandable that different authors have attained different results, even when working with the same species. In decapods, antennulae, antennae, mouth parts, tips of the appendages and the entire body surface have been variously described as the sites of chemoreceptors. Most of the evidence supports the special importance of the external ramus of the antennulae for the "distance" chemoreception, and of the mouth parts and other appendages for the contact chemoreception. The role of the receptors on the antennulae of *Callinectes* and *Cambarus* in chemoreception has also been proved through the electrophysiological studies of Hodgson, Lettwin and Roeder (1955) and Hodgson (1958).

As the removal of the antennae and antennulae in *Asellus* at first apparently abolished the reaction to NaCl, the chemoreceptors mediating the response seem generally to be situated on the antennae and/or antennulae. However, the results obtained for experiments performed one to three days after the amputation of the antennae and antennulae show that some sensitivity to NaCl may still exist in the animals or is rapidly regained.

More clear-cut results were obtained in the experiments with *Gammarus.* In these, the salinity receptors involved in reactions to brackish water are apparently on the antennulae and antennae. The removal of these also abolished the reaction to pure NaCl solutions. Thus, in amphipods the antennulae and antennae, and in isopods the antennulae and/or antennae, seem to contain the salinity receptors, and the results thus corroborate the various earlier observations on the location of the "distance" chemoreceptors in Crustacea.

It is tempting to think that "on terrestrial organisms the humidity has probably similar physiological effects as the salinity on aquatic organisms" (Kinne, 1957, p. 90). However, the problem faced by aquatic animals seems to be more in the maintenance of the proper ionic composition of their body fluids, while in terrestrial animals it is in the maintenance of a proper degree of dilution of the body fluids, already controlled for their ionic equilibrium. A more specific argument emerges from the present study: in *Asellus* the site of the salinity receptors is on the antennae and/or antennulae, which have recently been shown to be insignificant for the orientation of *Asellus* in an alternative chamber with different humidities of the air (Lagerspetz and Lehtonen, 1961).

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SUMMARY

1. The salinity reactions of *Asellus aquaticus* from fresh and Baltic brackish water (6‰), of *Idotea baltica* and of *Gammarus* spp. from brackish water were studied with the apparatus of Hodgson (1951). Both natural brackish-water and pure NaCl solutions were used.

2. None of the experimental animals had, for brackish water, reaction thresholds low enough to allow behavioral selection of salinities to occur in natural conditions.

3. The reaction threshold for NaCl solutions was higher in brackish-water than in fresh-water specimens of *Asellus*. *Asellus* always preferred the more dilute concentration. *Idotea baltica* and *Gammarus* preferred brackish water to fresh water. *Gammarus* preferred even 6‰ NaCl to fresh water.

4. In *Asellus*, the chemoreceptors mediating the response to salinity variations seem, for the most part, to be situated on the antennae and/or antennulae. In *Gammarus*, the salinity receptors are situated on the antennae and antennulae.

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A COMPARISON OF THE RESPONSES OF TRITURUS AND DESMOGNATHUS TO THYROID-STIMULATING HORMONE ADMINISTRATION [1]

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We have recently reported experiments comparing the effects of goitrogen treatment on the histology and the concentration of radioiodine by the thyroid glands of *Triturus viridescens* and *Desmognathus fuscus* (Dent and Lynn, 1958). The results indicate that, although these drugs markedly inhibit thyroidal concentration of iodine in both urodeles, characteristic histological changes in the gland occur in *Desmognathus* only. It was suggested that the failure of the *Triturus* thyroid to give the usual histological response to such treatment might be accounted for by either (1) some defect in the pituitary control of thyroid function, such that decreased thyroid hormone level in the blood does not cause any marked increase in TSH (thyroid-stimulating hormone) production, or (2) a lack of ability of the *Triturus* thyroid to respond to TSH stimulation. The present experiments were undertaken to test the latter alternative by study of the effects of administration of exogenous TSH upon histological structure and on radioiodine uptake in the thyroids of these two animals.

Materials and Methods

Specimens of *Triturus* (*Diemyctylus*) *viridescens viridescens* (Rafinesque) were collected from a pond near Monterey, Virginia. Specimens of *Desmognathus fuscus fuscus* (Rafinesque) were taken from a stream near Oliver Springs, Tennessee. The animals were kept in the laboratory for a minimum period of one month before being used for experiments. They were fed every other day, *Triturus* with ground beef fortified with cod liver oil and calcium phosphate, *Desmognathus* with live meal-worm larvae. Both before and during the experimental period the animals were kept in a constant temperature chamber at 23.0° ± 1.0° C.

Injections of 0.1 ml. of a solution of Armour's Thytropar (1.0 U.S.P. Unit per ml.) in 0.7% saline were made into the body cavities of the experimental animals on alternate days. The salamanders were always anesthetized in an aqueous solution of tricaine methane sulfonate (1 part in 1000) before injection.

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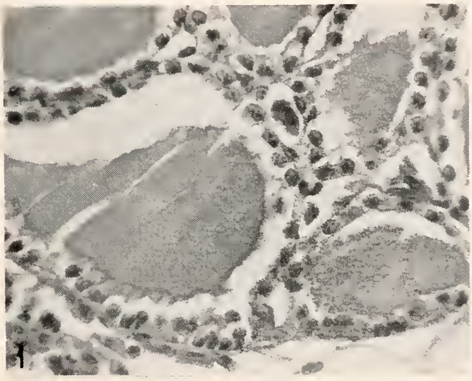
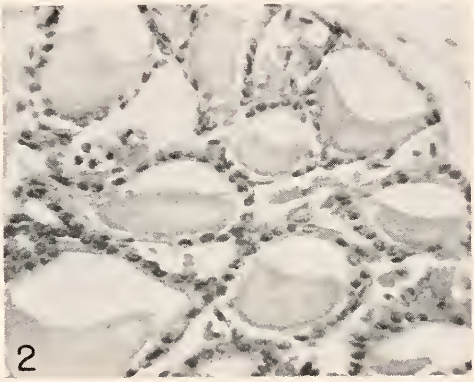
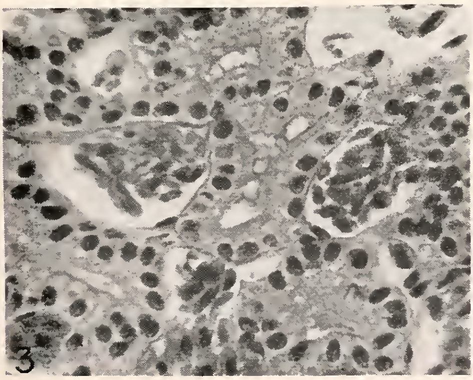
[4] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

For comparative study of the effects of injected TSH on the thyroids of *Triturus* and *Desmognathus*, groups of each species were given various numbers of injections and were killed various periods of time after the cessation of injection. The thyroid regions of these animals were fixed in equal parts of Bouin's fluid and ethyleneglycolmonoethyl ether. After sectioning they were stained with Harris' hematoxylin and Ponceau de Xylidine-Orange II (Gray, 1952).

Radioiodine uptake by and release from the thyroid was followed by a standardized procedure. Sixteen mature specimens of *Desmognathus* (2.8–3.3 g. body weight) and sixteen mature specimens of *Triturus* (2.5–3.3 g. body weight) were selected and divided into groups of eight controls and eight experimental animals. All animals were given six successive injections, experimental animals receiving the TSH preparation and controls receiving 0.1 ml. of 0.7% NaCl solution. Three hours after the last of these injections, each animal was given an additional intraperitoneal injection of 0.1 ml. of 0.7% saline containing 50.0 μc./ml. of I^{131}. At







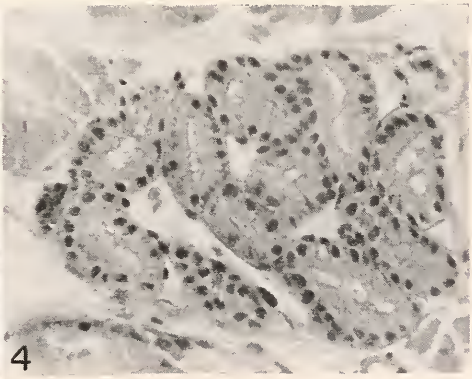


FIGURE 1. Section of thyroid gland of control specimen of *Triturus*.

FIGURE 2. Section of thyroid gland of control specimen of *Desmognathus*.

FIGURE 3. Section of thyroid gland of *Triturus* given seven successive injections of TSH on alternate days and killed 24 hours after the last injection.

FIGURE 4. Section of thyroid gland of *Desmognathus* given seven successive injections of TSH on alternate days and killed 24 hours after the last injection.

intervals of 6, 12, 24, 36, 48, and 60 hours thereafter,[5] each specimen was again anesthetized and the radioactivity of the thyroid and heart regions was measured by means of a previously developed technique (Dent and Lynn, 1958). In this technique a scintillation counter was used, consisting of a 1.5-inch NaI crystal cemented to the window of an RCA type 5819 photomultiplier tube and a conventional amplifier and binary scaler. The crystal and photomultiplier were mounted in a lead cylinder 5.2 cm. thick with a collimating slit measuring 4.0 by 12.0 mm. At the conclusion of the measurements of radioactivity, the animals were killed and their thyroids fixed and sectioned.

TABLE I

Radioactivity (corrected for physical decay) at the indicated periods of time after injection with 5 μc. of I^{131}. Experimental animals had previously received six successive injections of TSH. All means are based on observations on eight individuals.

$$\text{Standard deviation: } s = \sqrt{\sum_{i}^{n} \frac{(X_i - \bar{X})^2}{n-1}} \quad \text{where } n = 8.$$

Species	Time after I^{131} injection (hours)	Radioactivity (cts./sec.)			
		Controls		Treated	
		Thyroid	Heart	Thyroid	Heart
Triturus viridescens	6	4.43 ± 1.686	3.92 ± 1.074	5.71 ± 1.479	4.08 ± 0.856
	12	5.03 ± 1.201	3.01 ± 0.558	7.01 ± 2.224	2.74 ± 0.525
	24	4.88 ± 1.179	1.33 ± 0.694	9.41 ± 4.057	1.44 ± 0.659
	36	5.07 ± 0.962	1.16 ± 0.352	9.39 ± 3.807	1.20 ± 0.727
	48	4.93 ± 1.022	0.91 ± 0.259	9.97 ± 4.916	0.86 ± 0.493
	60	4.96 ± 1.105	0.70 ± 0.315	9.09 ± 3.805	0.90 ± 0.699
Desmognathus fuscus	6	4.29 ± 1.040	6.14 ± 1.724	7.65 ± 1.492	5.11 ± 1.726
	12	5.35 ± 1.004	4.83 ± 0.812	6.16 ± 1.731	3.66 ± 1.327
	24	4.56 ± 0.765	3.24 ± 0.702	4.44 ± 1.452	2.57 ± 0.581
	36	3.75 ± 0.645	2.10 ± 0.579	3.97 ± 1.391	1.83 ± 0.580
	48	3.26 ± 0.569	1.62 ± 0.212	3.60 ± 1.930	1.68 ± 0.311
	60	3.02 ± 0.977	1.45 ± 0.215	3.16 ± 1.431	1.42 ± 0.403

RESULTS

1. *Effects of TSH treatment on thyroid histology*

It has previously been pointed out (Dent and Lynn, 1958) that, during the summer months, the thyroids of untreated *Triturus* present a quite uniform histological appearance characteristic of relatively low activity. The follicles are well rounded, the epithelium is low, the colloid is homogeneous and acidophilic, chromophobe droplets are absent or sparse (Fig. 1). The thyroids of untreated *Des-*

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[5] In preliminary experiments, radioactivity measurements were made at hourly intervals for the first 8 hours after injection of radioiodine. It was found that the activity in the thyroid reached peak levels at 5–6 hours in *Desmognathus* but was still rising steadily in *Triturus* at 8 hours. In all later experiments the measurements were begun at 6 hours and continued for 60 hours at the intervals indicated.

mognathus, on the other hand, show more individual variation but all present an appearance of moderate to relatively high activity: cuboidal to columnar epithelium; less homogeneous, often basophilic, colloid; relatively abundant chromophobe droplets at the peripheries of the colloid masses (Fig. 2).

Administration of TSH caused marked changes in the thyroid in both. Thyroids of animals given seven injections (over a 13-day period) and fixed 24 hours after the last injection showed the maximal effects seen in this experiment and have been chosen for illustration (Figs. 3 and 4). It will be noted that, in both *Triturus* and *Desmognathus,* the epithelium increased greatly in height, the colloid was almost entirely discharged from the gland, and the follicles became irregular in shape. Study of the thyroids of animals given smaller numbers of injections

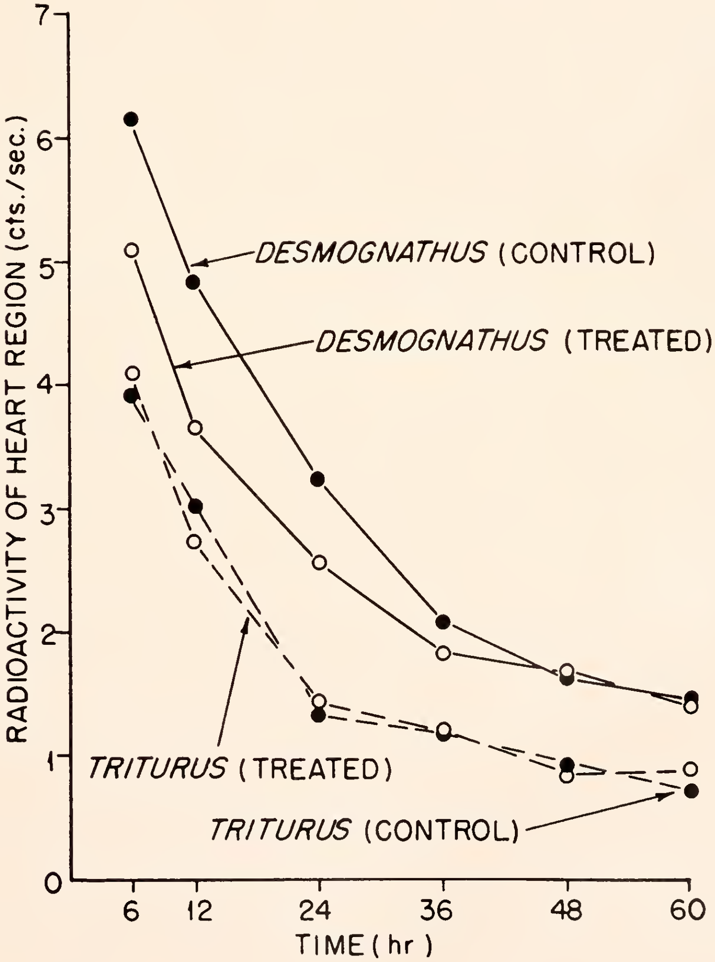

FIGURE 5. Radioactivity in the heart regions of experimental and control specimens of *Desmognathus* and *Triturus* over a period of 60 hours after administration of radioiodine.

shows that these same effects are obtained with six, five, or four injections. Thyroids of salamanders given three or two injections showed less marked but definite histological responses. Thyroids of animals that received a single injection could not be distinguished from those of controls.

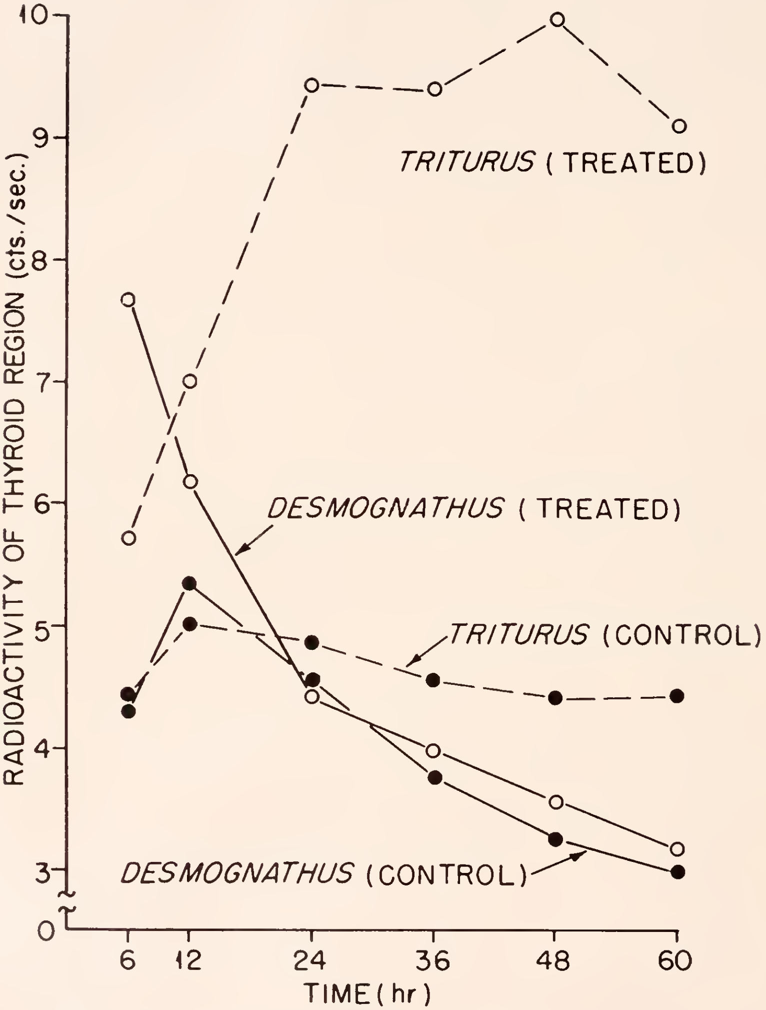

FIGURE 6. Radioactivity in the thyroid regions of experimental and control specimens of *Desmognathus* and *Triturus* over a period of 60 hours after administration of radioiodine.

By giving a sufficient number of injections of TSH to ensure marked responses (4–8 injections), and varying the times of fixation after the last injection, it was ascertained that the maximal histological response persists for 36–48 hours. Thyroids of specimens fixed 60 hours after the last TSH administration showed definite indication of regression toward the normal histology with a decrease in epithelial height and the beginning of accumulation of stored colloid. By 75 hours regression was well under way and by 144 hours the thyroids of treated salamanders were like those of controls.

2. *Effects of TSH treatment on uptake of radioiodine by the thyroid*

The means of counts of radioactivity (corrected for physical decay) in thyroid and heart regions of control and experimental animals at successive periods after injection of I^{131} are shown in Table I, and these data are illustrated graphically in Figures 5 and 6.

It will be noted (Fig. 5) that the heart region, which we consider as representative of soft tissues other than those of the thyroid, shows relatively high counts at 6 hours after I^{131} administration in both control and experimental groups of both species. The counts then decline rapidly as the radioiodine is eliminated from the circulating blood. There is no significant difference between experimental and control groups within the species, but the counts for the *Triturus* heart region are uniformly lower than those for *Desmognathus.*

Counts for the thyroid region (Fig. 6) reveal that treatment with TSH results in an increased uptake of radioiodine by the gland in both *Triturus* and *Desmognathus.* At 6 hours after I^{131} injection, the counts in the thyroid regions of both groups of experimental animals are well above those in the corresponding controls. However, the later counts show important differences between the two species. In *Desmognathus,* the counts decline quickly and in 24 hours have reached the level of the control thyroids. Thus, the initial rate of uptake of radioiodine is high but the rate of turnover is also high and there is no significant retention of I^{131} in the thyroids of experimental animals as compared with those of controls after 12 hours. In *Triturus,* on the other hand, the counts rise more slowly in the period up to 24 hours after I^{131} injection and then level off with little sign of decline by the end of the experiment (60 hours). In this species, then, the TSH treatment caused increase in radioiodine uptake and this, combined with a slow rate of turnover, resulted in retention of I^{131} at a much higher level than in the controls. It may be noted that the curve for the thyroids of *Triturus* control specimens shows little change in counts throughout the counting period. This indicates the relatively low rate of activity of the thyroid in normal animals of *Triturus* which we have reported previously (Dent and Lynn, 1958).

DISCUSSION

Our earlier experiments indicated that, although both *Triturus* and *Desmognathus* show a marked inhibition of thyroid function after treatment with goitrogenic drugs, the usual histological changes that occur after goitrogen treatment are not seen in *Triturus* during treatment periods of less than two months duration.[6] These histological changes are normally induced by an increased production of pituitary thyrotropin after a decrease in the level of circulating thyroid hormone. The absence of such changes in the *Triturus* thyroid could indicate either a failure of the pituitary to produce significantly higher levels of thyrotropin after thyroid inhibition or a failure of the thyroid to respond in the typical way to thyrotropin stimulation.

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[6] Adams, 1946a, and Dent (unpublished) have shown that the thyroid of *Triturus* undergoes hyperplasia after several months of treatment with goitrogens.

The results reported here show clearly that the *Triturus* thyroid is capable of responding to exogenous thyrotropin. The histological response of the *Triturus* thyroid proved to be entirely comparable to that of the *Desmognathus* thyroid and the effect of TSH treatment on radioiodine uptake was even more marked in the former than in the latter. These findings thus indicate that the histological signs of low thyroid activity in the normal gland of *Triturus*, and its failure to show signs of increased activity after goitrogen treatment, are to be attributed to a low level of thyrotropin production.

The fact that treatment with exogenous TSH causes such a marked increase in radioiodine uptake in *Triturus* as compared with *Desmognathus* may be taken as further evidence that TSH production is unusually low in *Triturus*. Several previous workers have concluded that the responsiveness of the thyroid to exogenous TSH varies inversely with the thyrotropin content or production of the animal's own pituitary (Loeb and Friedman, 1930; Collip and Anderson, 1935). Indeed, the selection of the young guinea pig and the chick as favorable animals for assay of thyrotropin is based on the relatively low thyrotropin content of the pituitary in these animals and the accompanying high sensitivity of their thyroids (Adams, 1946b). Among cold-blooded vertebrates the goldfish (*Carassius auratus*) is the best known example of a species in which the thyroid normally presents a histological appearance of very low activity. The goldfish thyroid, like that of *Triturus*, shows no histological evidence of increased activity after goitrogen administration (Fortune, 1955) but responds strongly to injected TSH (Gorbman, 1940; Berg and Gorbman, 1954). Fortune (1956) has suggested that in the goldfish the inactive thyroid, resulting from absence or very low level of TSH production, may be an important factor in this animal's exceptional ability to withstand high temperature. We have previously pointed out (Dent and Lynn, 1958) that this hypothesis may be valid for *Triturus* as well, since this salamander also has a high upper limit of temperature tolerance.

We wish to acknowledge the assistance of Dr. M. A. Kastenbaum of the Oak Ridge National Laboratory Mathematics Panel in the statistical analysis.

Summary

1. Specimens of *Triturus viridescens* and *Desmognathus fuscus* were given injections of Armour's Thytropar on alternate days.

2. Histological examination of the thyroid glands of experimental and control animals showed that both species gave maximal response after only four injections, the follicular epithelium being greatly heightened and intrafollicular colloid almost completely discharged, and that the response persisted for 3–4 days after cessation of treatment, but then declined and disappeared by 7 days.

3. Measurements of uptake and turnover of I^{131} by the thyroids of animals given six injections of thyrotropin showed a high uptake and a rapid turnover in *Desmognathus*. In *Triturus* the effect of the treatment upon I^{131} uptake was also

marked and the radioiodine was retained at a high level throughout the counting period (60 hours).

4. It is concluded that the *Triturus* thyroid is more responsive to exogenous TSH than is that of *Desmognathus*, and that this is related to an exceptionally low TSH content and production in the anterior pituitary of *Triturus*.

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SYSTEMATICS AND DISTRIBUTION OF AN ESTUARINE ISOPOD CRUSTACEAN, CYATHURA POLITA (STIMPSON, 1855), NEW COMB., FROM THE GULF AND ATLANTIC SEABOARD OF THE UNITED STATES

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In estuaries and tidal marshes along the southern and eastern seaboard of the United States, there lives a burrowing anthurid isopod of the genus *Cyathura*. It is the only species of this genus thus far reported over a long range extending from Lake Pontchartrain, Louisiana to Chewonki Creek, Maine. It has long been misidentified as *Cyathura carinata* (Krøyer), a species originally described from Copenhagen Harbor, Denmark, and subsequently reported from many other localities in Europe, Greenland, the Mediterranean, Africa, and Asiatic Russia. In this paper, evidence will be presented to show that the eastern American form is specifically distinct from *C. carinata,* and it will be redescribed under its proper name.

The ecology and ecological distribution of our east coast *Cyathura* have been investigated by one of the present authors (Burbanck *et al.*, 1956; Burbanck and Burbanck, 1958; and Burbanck, 1959a, 1959b), while the other (M.A.M.) has been mainly concerned with the systematics and zoögeography of this and related species. The preceding statement indicates the general areas of responsibility of the authors of this article.

American material for this study was obtained from the U. S. National Museum through the courtesy of Drs. Fenner Chace and T. E. Bowman, amply supplemented by W. D. Burbanck's extensive collections of eastern American *Cyathura* from its known range. From South Africa, Dr. K. H. Barnard kindly sent paratypes of *Cyathura estuarius* Barnard (1914) which he later (1925) assigned to the synonomy of *C. carinata* (Krøyer). European material from the type locality and elsewhere was graciously supplied by Drs. Torben Wolff, A. Panning and E. Rasmussen. The authors take this opportunity to acknowledge the valuable assistance of these esteemed contributors. We also wish to thank many others whose names and assistance are mentioned in the text. Finally, we are grateful to Dr. G. Victor Morejohn for the illustrations and technical assistance.

The nomenclatorial history of the eastern American *Cyathura* has been quite confused. It was first mentioned in the literature under the name *Anthura gracilis* Montagu by Gould (1841) and later by DeKay (1844) in faunal accounts of Massachusetts and New York, respectively. Subsequently it was twice described as a new species—first, by Stimpson (1855) under the name *A. polita*, and nearly twenty years later by Harger (in Verrill, 1873) under the name *A. brunnea.* In 1878, however, Harger (1880) rightly relegated his species to the synonomy of Stimpson's earlier described *A. polita.* At the same time, he noted that the American forms approached the descriptions and figures of *A. carinata* Krøyer, but he also noted many discrepancies and regretted the lack of European specimens for comparison.

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Although Harger did observe a resemblance between the European and American forms, he apparently did not regard them as conspecific. As a matter of record, Harger (1879) considered *Anthura polita* to be (p. 162) "A southern species, not found north of Cape Cod until the summer of 1878, when it was taken at Gloucester, Mass."

In 1886, Norman and Stebbing founded the genus *Cyathura* for *Anthura carinata* Krøyer. They formally placed *A. gracilis,* DeKay (*non* Montagu), *A. polita* Stimpson, and *A. brunnea* Harger in the synonomy of *Cyathura carinata* (Krøyer), thus establishing *Cyathura* as a monotypic genus. Unfortunately, they based the description of the new genus on American material which they mistakenly considered to be conspecific with *C. carinata.* "Our description," they state (p. 125), "is drawn from specimens kindly sent to us, named *Anthura brunnea,* by Mr. S. I. Smith, the talented carcinologist of Yale College. There can be no doubt, we think that it is the *A. carinata* of Kröyer." They dismissed Harger's mention of disagreements between American specimens and Krøyer's (1847 and 1849) description with the statement that (p. 125) ". . . if the figures of the parts so described which are given in the 'Voyage en Scandinavia' &c be examined the apparent discrepancies seem to disappear." Had careful comparisons been made by these early authors between representative European and American specimens, it is likely that the differences, which we note later, would have been observed. As it is, the key source of the subsequent confusion was their compound error, first in placing the American species in the synonomy of *A. carinata* Krøyer, the type of their new genus *Cyathura,* and then basing their genotypic description on the misidentified American specimens.

Richardson (1900, 1905) naturally followed Norman and Stebbing's disposition which until now has never been seriously questioned. In her monograph (1905), Richardson noted that three specimens—two from Florida and one from South Carolina—differed in certain respects from other specimens, but she did not regard these variations as particularly significant.

The subsequent general acceptance of the conclusion that the eastern American form is conspecific with *Cyathura carinata* (Krøyer) is not surprising in view of the eminence of the above-mentioned authorities, the misidentified "genotype," and the superficial similarity of many anthurid species, coupled with the inadequacy of early descriptions and illustrations. Moreover, a rather intriguing zoögeographical picture emerged when eastern North America was added to the previously known records of *C. carinata,* for this seemed to extend its distribution in an arc around the North Atlantic basin. Allee's (1923, p. 179) listing of it as a "north ranging species" and its reported occurrence in Greenland are in line with this notion. Indeed, the question has been raised whether this species might not even be completely circum-Atlantic or even cosmopolitan in distribution. It has been reported from the western and southern coasts of Africa (Monod, 1925; Barnard, 1925; Day *et al.*, 1952). No cyathurans have been reported, however, from the Atlantic coasts of South America, but the possibility of their occurrence there cannot be dismissed inasmuch as the shores of that continent have been inadequately explored for isopods. Mention should be made here of a West Indian species, *C. crucis* Barnard (1925), taken from a depth of four fathoms at St. Croix, Virgin Islands, and of another Caribbean species, *C. curassavica* Stork (1940) from Curacao in the Netherland Antilles.

The larger concept of world-wide distribution of *Cyathura carinata* (K.) obviously results from additional reports of this species in the Mediterranean (Stammer, 1932; Larwood, 1940), China (Tattersall, 1921), and the Okhotsk Sea (Gurjanova, 1936), as well as many references to it in Western Europe. It has not been found, however, in the entire east Pacific. Another species, *C. munda* Menzies (1951), occurs along the California coast from Marin County to the Mexican border (Menzies and Barnard, 1959), but its distributional limits have not yet been established.

An alternative zoögeographical hypothesis suggested by Burbanck (1959b, p. 508) is that the several species of *Cyathura* in the northern hemisphere, including our eastern American forms, and the Eurasian *C. carinata*, ". . . may have arisen from a common preglacial species which had a circumpolar distribution. When the original species was subdivided once or many times by lobes of glaciers during the last ice age and driven south, speciation may have occurred. Now during the present interglacial period, populations of *C. carinata* (K.) and the North American *Cyathura* sp. may be moving north again." As evidence, Burbanck cites: (1) the absence of cyathurans in Norway; (2) reports of the appearance of *C. carinata* in Sweden in the 1930's (Löwegren, 1937; Lundstrom, 1937); (3) Harger's statement that the American form is a southern species that got only as far north as Gloucester, Mass., in 1878; and (4) the unsuccessful attempts of himself and others to find cyathurans north of Maine in the Gulf of St. Lawrence, Baie des Chaleurs, Cape Breton Island, and Mount Desert Island. Furthermore, the Greenland report must be discounted since Dr. Torben Wolff (personal communication) has been unable to find any authentic record of *C. carinata* from Greenland. It is apparently either a case of mistaken identification or an error in the literature. Thus there appears to be a long gap between American and European cyathurans, which is consistent with Burbanck's hypothesis.

The present authors agree with the generic designation, but question the specific determination of the eastern American cyathurans as *Cyathura carinata* (Krøyer) on two grounds. First, there are some disturbing discrepancies, both between descriptions and, more importantly, between specimens of *C. carinata* collected in Europe (including the type locality and vicinity), on the one hand, and our American specimens on the other. These differences will be discussed later. Secondly, on *a priori* grounds, it seems inconceivable that a species apparently limited in its habitat requirements to estuarine conditions (Burbanck, 1959a, 1959b) could become so widely dispersed without undergoing speciation as a consequence of ecological segregation and hence reproductive isolation. (Indeed, on the same theoretical basis, one might even expect some evolutionary divergence to have occurred in the presumably segregated cyathuran populations along the entire eastern seaboard or at least at the extremes of the range, assuming sufficiently long periods of isolation.)

Pending the present systematic revision, the authors have deemed it advisable to refer to the eastern American forms as *Cyathura* sp. The genus is certainly correct, as the species fits the original brief generic diagnosis given by Norman and Stebbing (1886, p. 121) and, of course, their description of the genotype since that was based on American specimens. It also fits the longer description (quoted below) of the genus given by Barnard (1925, p. 139) in his revision of the Anthuridae.

Genus Cyathura Norman and Stebbing, 1886

"Eyes typically present, sometimes absent. Peraeon typically with dorsolateral keels and dorsal pits. Pleon with sutures indistinct dorsally. Telson lenticular in cross-section, thin, smooth. Antenna 1 with flagellum 1-3-jointed, sometimes brush-like in ♂. Antenna 2 with flagellum of a single joint. Mandible with 3rd palpal joint usually larger than 1st, with rather large apical tuft of setae. Maxilliped 4-jointed [counting a basal joint anchylosed to head]. Peraeopod 1 with more or less pronounced tooth on palm of 6th joint, unguis typically long. Peraeopods 2 and 3 with 6th joint cylindrical. Peraeopods 4–7 with 5th joint underriding 6th. Pleopod 1 not indurated. Uropods not indurated. Exopod folding over telson. Oostegites 3 pairs (in *siamensis* and also, *apud* Harger, in *"Anthura polita"* = *C. carinata*)."

Despite the fact that the type species was described from American specimens erroneously thought to be identical with it, there is no point in designating the American species as the new genotype since Norman and Stebbing's intention was clear to establish *Anthura carinata* Krøyer as the type by monotypy. The name, *"Cyathura carinata* (Kröyer)," has line precedence to the description and, in our opinion, represents the valid genotype. For its description, the original one of Krøyer (1847) and those of subsequent writers (*e.g.* Schiödte, 1875, 1876) may be consulted.

Including the type species, thirteen species of *Cyathura* have been described, but, as indicated in the following list, some have or possibly should be transferred to other genera.

Name	Distribution
Cyathura carinata (Krøyer, 1847) (type species)	Widely distributed in Europe, etc.; see text
C. crucis Barnard, 1925	West Indies
C. curassavica Stork, 1940	Netherland Antilles
C. eremophila Monod, 1925	Mauritiana, West Africa
C. estuarius Barnard, 1914 (= *C. carinata*, Barnard, 1925)[3]	South Africa
C. indica Barnard, 1925	Singapore, Siam, Paumben, Quilon, Travancore (Barnard, 1935)
C. liouvillei Monod, 1925 (= *Anthelura*?)	West Africa

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[3] Comparison of paratypes (all female) of *C. estuarius*, kindly sent by K. H. Barnard, with European specimens of *C. carinata* indicates that he may have been mistaken in assigning the former to the synonomy of the latter. Examination of adult males from South Africa would be needed to determine whether or not *C. estuarius* should be resurrected.

Name	Distribution
C. milloti Chappuis, Delamare Deboutteville, and Paulian, 1956	Reunion, Madagascar
C. munda Menzies, 1951	Central California
C. pusilla Stebbing, 1904 (= *C. indica* Barnard, 1935)	Ceylon and British East Africa
C. robertiana Monod, 1925 (= *Anthelura robertiana*, Monod, 1925)	Morocco
C. siamensis Barnard, 1925	Siam
C. truncata Hansen, 1916 (= *Anthelura truncata*, Barnard, 1925; and Monod, 1925)	Davis Strait, Canada

We now propose to add a fourteenth name to the list by removing Stimpson's *Anthura polita* from the synonomy of *Cyathura carinata* (K.) and reestablishing it as a distinct species in the genus *Cyathura*. From the preceding discussion, it is apparent that Stimpson's name is not only available but appropriate in the new combination as follows.

Cyathura polita (Stimpson, 1855), New Combination

Synonomy

Anthura gracilis, De Kay, 1844, p. 44, pl. 9, fig. 34 (*non A. gracilis* Montagu).
Anthura polita Stimpson, 1855, p. 393; Harger, 1879, p. 162; Harger, 1880, pp. 398–402, pl. 11.
Anthura brunnea Harger, 1873, pp. 426, 428, 572–573.
Cyathura carinata, Norman and Stebbing, 1886 (*in partem, non Cyathura carinata* [Krøyer]); Richardson, 1900, p. 215; Richardson, 1905, pp. 64–66, figs. 47–50; Burbanck *et al.,* 1956, esp. pp. 236–237; Burbanck and Burbanck, 1958, p. 346; Burbanck, 1959a, p. 22; Burbanck, 1959b, pp. 507–511.

(Other references to *C. carinata* along the eastern coast of the United States are doubtless referable to *C. polita.*)

Description

Stimpson's (1855) original description of *Anthura polita* (= *Cyathura polita*) follows (p. 393).

"Cylindrical, smooth and shining; the seventh segment nearly as large as the sixth. Head small, inferior antennae as long as the head, somewhat larger than the superior ones and placed before them; eyes very minute, black, placed rather on the sides of the head at the anterior corners. Legs of the first pair very thick, the rest slender. Abdomen short and broad. Color pale greyish, mottled. Length, 0.9 inch; breadth, 0.13 inch. Found at the depth of two inches in sand, above half-tide.

"*Hab.* Coast of the United States, at Norfolk."

The above cited description of *Anthura polita* and Harger's (1873) description of *A. brunnea,* its first synonym, are obviously much too brief and generalized for comparative purposes. Richardson's (1905) and Norman and Stebbing's accounts of the eastern American form under the name *Cyathura carinata* (Krøyer) are somewhat better but lack the critical details which differentiate *C. polita* from *C. carinata*. Little could be gained by comparing American material with these early acounts or with more adequate descriptions of true *carinata* given by Krøyer (1847, original description), Schiödte (1875, 1876), Barnard (1925), and Stephensen (1948).

Table I

Comparison of Cyathura carinata *(Krøyer) from Europe and* C. polita *(Stimpson) from eastern United States*

Characteristics	*Cyathura carinata*	*Cyathura polita*
Body Length (Average)	12.3 mm. (5 spec., Dybso Fjord) 9.5 mm. (2 ♀♀, Copenhagen Harbor) 10.5 mm. (1 ♀, Plymouth, England)	18.0 mm. (5 spec., Salt Springs, Fla.) 18.4 mm. (10 spec., Stony Brook, Mass.)
Head and Appendages		
General (Fig. 1)	Median rostral point bluntly pointed. Eyes small.	Median rostral point more truncate. Eyes small.
Antenna 1 (Fig. 2)	No distinctive differences between European and American. Flagellum brush-like in males of both species.	
Antenna 2 (Fig. 2)	Grooved 2d article, with small spines near distal inner edge. 1½ times longer in ♂ than ♀.	Grooved 2d article, without small thorn-like setae near inner margin. Subequal in length in ♂ and ♀.
Mandible (Fig. 3)	Four to 6 stout setae on 3d article of palp; 2–3 long setae on 2d article of palp; 18–20 serrations on flattened cutting flange.	Thirteen to 14 stout setae on 3d article of palp; 5–7 setae on 2d article of palp; 14–18 serrations on flattened cutting flange.
Maxilla 1 (Fig. 4)	Outer lamina: with 1 large and 7 small apical teeth. Collar of subterminal bristles encircles lamina.	Outer lamina: with 1 large and 6 small apical teeth. Subterminal bristles on inner and outer edges.
	Inner lamina: No essential difference between European and American species. Relatively small with single apical spine.	
Maxilla 2 (?) + *hypopharynx* (Fig. 4)	No distinctive differences between European and American species in this complex.	
Maxilliped (Fig. 4)	Three-jointed + coalesced basal piece; essentially similar in both species.	
Pereion and Pereiopods		
Pereion	Similar in both species. First segment longest, seventh shortest. Broadly V-shaped or carinate ventrally.	
Epimera	Visible along lateral margins of all segments in both species.	
Pereiopod 1 (*Gnathopod*) (Fig. 5)	Sexually dimorphic. Propodus in ♂ slender with proximal lateral margin flattened and curved outward; propodus in ♀ swollen with no outcurved proximal edge. Palm toothed in both sexes.	Not sexually dimorphic. Propodus in both sexes with palmar tooth.

TABLE 1—*Continued*

Characteristics	*Cyathura carinata*	*Cyathura polita*
Pereiopod 6 (Fig. 5)	Propodus length three times its width in both sexes.	Propodus length four times its width in both sexes.
Pleon, Telson and Appendages		
Pleon (Fig. 6)	First five pleonites fused, with partial lateral suture and indentation on anterolateral side indicating first pleonite. Paired lengthwise sutures diverge anteriorly, ending in circular area with concentric rings of attachment of pleopod muscles.	Same, except anterolateral sutures and indentation less pronounced in southern specimens. Occasional specimens show additional faint lateral sutures.
	Sixth pleonite free along entire anterior and posterior border, the latter incised middorsally.	Sixth pleonite free along anterior and posterior border except where the latter fuses posteriorly with telson on each side of middorsal line.
Pleopod 2 ♂ (Fig. 7)	Terminal complex of appendix masculinum extends well beyond rounded apical edge of endopod. Hook-crowned rod originates in angle between base of lateral lobe and tip. Tip does not extend beyond end of hooked rod.	Terminal complex of appendix masculinum does not extend significantly beyond apical edge of endopod which is obtusely produced distomedially beyond lateral lobe. Hook-crowned rod originates some distance from base of lateral lobe. Distal end of appendix extends beyond end of hooked rod.
Uropods (Figs. 1 and 4)	Outer distal margin of exopod distinctly incised.	Outer distal margin of exopod not distinctly incised, almost entire.
Telson (Figs. 1 and 6)	Sides converging posteriorly toward broadly rounded apex.	Sides subparallel anteriorly but converging posteriorly into broadly rounded apex.

Paired statocysts present near base of telson in both species.

Much more useful were part-by-part comparisons of representative American and European specimens, supplemented by reference to the literature. Therefore, it seems best to redescribe *C. polita* largely on a comparative basis emphasizing the characters that differentiate it from *C. carinata*. In lieu of type material of either form, European specimens unmistakably identified as *C. carinata* (K.) were compared with our eastern American *C. polita*. The European material included specimens from the type locality (Copenhagen Harbor) and vicinity (Dybso Fjord, Insel Fehmarn, Ulfsund), and Plymouth, England. The American material comprised extensive collections made by Burbanck along the eastern American sea-coast in connection with his ecological studies, and the American collections labelled *"Cyathura carinata"* in the U. S. National Museum, kindly made available to us through Dr. Fenner Chace. To facilitate the study, representative specimens of both sexes were selected from two European and two American localities for complete dissection and part-by-part comparison, but specimens from other localities were also included.

The analyses involved not only comparisons of previously described characters, but also attempt was made to find others of systematic significance. In the latter category, the appendix masculinum of the second pleopod of the mature male deserves special mention. This structure has been described in *C. carinata* and other anthurids by various authors (*e.g.* Omer Cooper, 1916; Barnard, 1925), but surprisingly little systematic use has been made of it in this group. The taxonomic importance of inherently stable genital structures is generally recognized since they presumably are not subject to environmental modification, and may, in some instances, actually determine by their structural conformations whether or not interbreeding can take place. As will be seen, the appendix masculinum serves as a valuable diagnostic character in the present study. Another objective was to determine the degree of intraspecific variation in taxonomic characters as this relates directly to their reliability.

The results of the comparison are shown in Table I and Figures 1–7. Although many of the differences indicated seem rather minor, they are consistent and collectively impressive. The major distinctions are: (1) size, (2) the many differences in detail of the mouthparts, (3) the sexual dimorphism in the gnathopods of the European species and the lack of it in the American form, (4) the difference in the articulation of the telson with the sixth pleonite in the two forms, (5) the shape of the telson, (6) the deeper incised exopod of the uropod in *Cyathura carinata*, and (7) the differences in the appendix masculinum.

As to size, *Cyathura polita* seems considerably longer than *C. carinata*, judging from available specimens. Our sample of the latter, however, is too small for a fair comparison. In the literature, *C. carinata* is reported as ranging up to 27 mm. in length. The range in length of the eight specimens of *C. carinata* that we examined was 9.3–14.2 mm., whereas the measured samples of *C. polita* ranged from 15.2 to 20.2 mm. (Salt Springs, Fla.) and from 14 to 21 mm. (Stony Brook, Mass.). The two largest specimens of *C. polita* in our collection are a female from Silver Glen Springs, Lake George, Fla., which is 23 mm. in length, and a male from Pocasset River, Mass., which is 25 mm. long. A statistical comparison of random samples of adults of the two species would be needed to determine whether there actually is a significant difference in size between the two species, but present data certainly indicate that *C. polita* is larger than *C. carinata*.

Regarding mouthparts, perhaps the most important differences are seen in the mandibles. There is much greater setation of the second and third articles of the palp in American as compared to European forms, and fewer teeth on the serrated, subapical cutting lobe in American than in European forms. The post-mandibular mouthparts also exhibit differences.

The sexual dimorphism in the propodus of the gnathopod in *Cyathura carinata* has not hitherto been reported. The lack of it in American cyathurans comprises an important diagnostic characteristic.

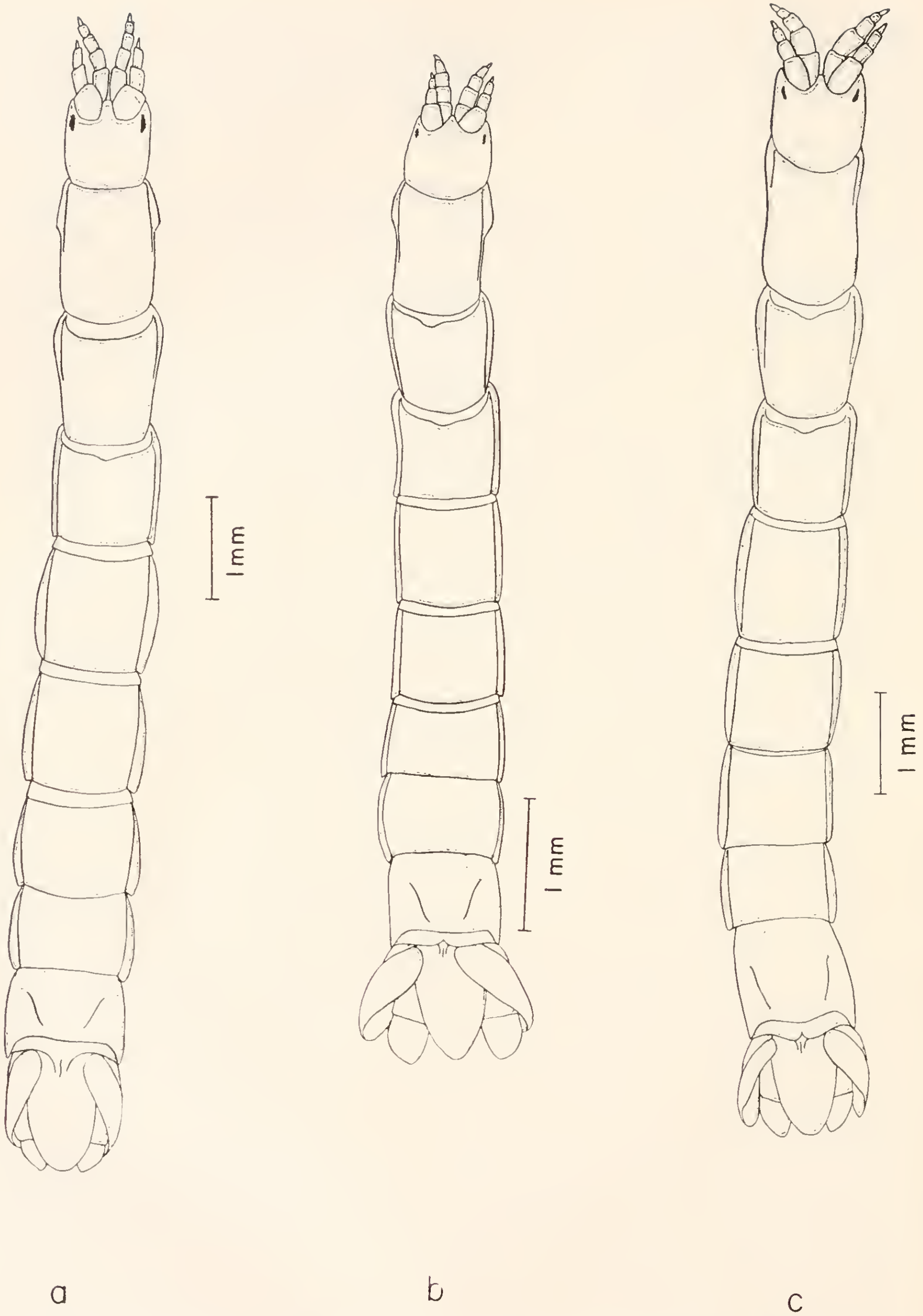

FIGURE 1. American *Cyathura polita* (Stimpson) and European *C. carinata* (Krøyer), dorsal views of females (setae, pigmentation, and pereiopods omitted for clarity) from Suwanee River, Fla. (a); Copenhagen Harbor, Denmark (b); and Plymouth, England (c).

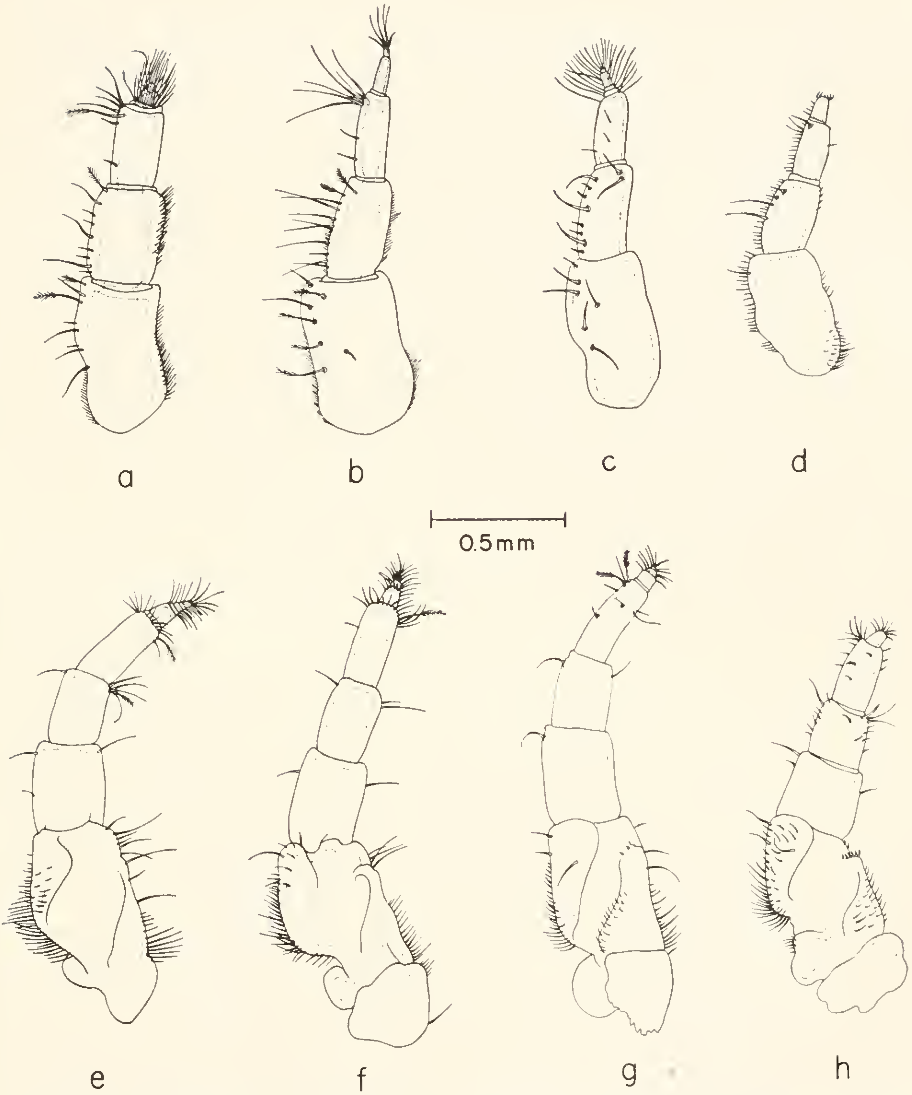

FIGURE 2. Antennae of *Cyathura polita* (Stimp.) from Suwanee River, Fla., and European *C. carinata* (K.) from Dybso Fjord, Denmark. First antennae: *C. polita*—(a) ♂, (b) ♀; *C. carinata*—(c) ♂, (d) ♀. Second antennae: *C polita*—(e) ♂, (f) ♀; *C. carinata*—(g) ♂, (h) ♀.

The tabulated differences between *Cyathura carinata* and *C. polita* in the articulation of the telson with the pleon, in the shape of the telson, and in the apical incision of the uropodal exopod are fairly easily recognizable, given the proper view. The articulation of the telson with the free sixth pleonite may be obscured in the critical middorsal area by a heavy fringe of setae along the posterior border of the pleon, but can be seen if these are removed (Figs. 6a and 6c). The lateral

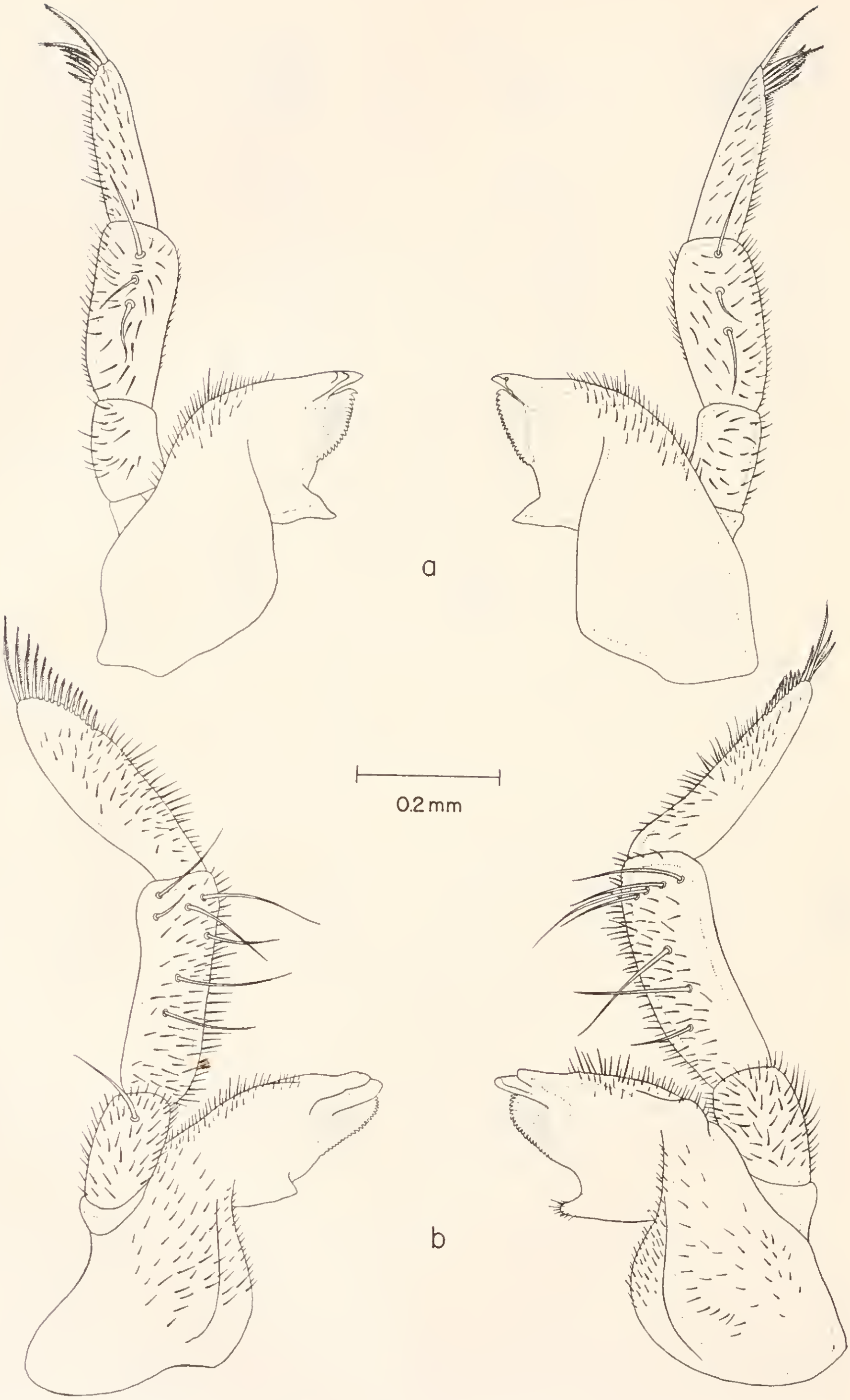

FIGURE 3. Mandibles of *Cyathura carinata* (K.) from Dybso Fjord, Denmark (a); and *C. polita* (Stimp.) from Lake George, Fla. (b).

margins of the telson may be covered anteriorly by the exopod of the uropod, but they can readily be observed if the exopods are spread apart. Then the subparallel sides of the telson in *C. polita* may be contrasted with the tapering margins of the telson in *C. carinata* (Figs. 6a and 6d). The deep apical incision of the exopod of the uropod in *C. carinata* (Figs. 6f and 6g), which is slight or absent in *C. polita* (Fig. 6e), can best be seen in lateral view.

Since the appendix masculinum has not hitherto been used extensively as a diagnostic characteristic, additional comments concerning it are in order. Attention was first called to this structure by Omer Cooper (1916) who described it from males of *Cyathura carinata* (K.) taken from brackish water in Christchurch Harbour, Hants. Subsequently, Barnard (1925) gave figures of it for several other anthurids. Presumably, these appendices serve as copulatory organs (as in isopods generally), but the details of the process and the function of their constituent parts are unknown. It develops along the inner edge of the endopod of the second pleopod of maturing males, becoming separated from it as a long, hollow, cylindrical structure, probably in the course of one or more molts. Successive stages in its separation are indicated by a series of specimens shown in Figure 7 (u, v, w). In some anthurids, it may extend far beyond the distal margin of the endopod, as in *C. milloti* Chappius, Deboutteville and Paulian and *Pseudanthura lateralis* Richardson, in which two species it appears to be about twice the length of the endopod. It is distinctly jointed about a third to a half of its length from the base.

The apex varies among different anthurids. It is simple and club-shaped in *Cyathura munda* Menzies; slender, pointed and plumose in *Calathura brachiata* (Stimpson); slightly recurved at the tip in *Cyathura crucis* Barnard; strongly recurved like a crochet hook in *Pseudanthura lateralis* Richardson; with a lateral apophysis coiled like a ram's horn in *C. milloti* Chappius, Deboutteville and Paulian; provided with a laterally-projecting, subterminal lobe in *Accalathura crenulata* (Richardson); and the most complex of all in *C. carinata* (Krøyer) and *C. polita* (Stimpson). In the latter two, there is a subterminal apophysis as in *A. crenulata,* but this bears in addition a hollow rod-like process capped with a crown or recurved teeth (Fig. 7, a–r).

Although the sexual stylets of European and eastern American cyathurans are constructed on the same basic plan, there are some significant differences in detail. Our observations on European *Cyathura carinata* agree with those of Omer Cooper (1916) that the appendix masculinum in that species extends well beyond the distal edge of the endopod and that the hook-crowned rod extends beyond the tip of the stylet. They do not agree, however, on the point of origin of the rod, or on the shape of the end of the subterminal lobe. According to Omer Cooper's descriptions and figures, the rod springs from the center of the flattened, subterminal lobe which is squarish at the tip. According to our observations of this structure in several males from Denmark, however, the rod originates at the base of the subterminal lobe which is bluntly rounded, rather than truncate, at the tip. The rod of the appendix in *C. polita* males, on the other hand, always originates a significant distance away from the point of origin of the subterminal lobe—anywhere from a quarter to three-quarters of the distance from the base of the lobe

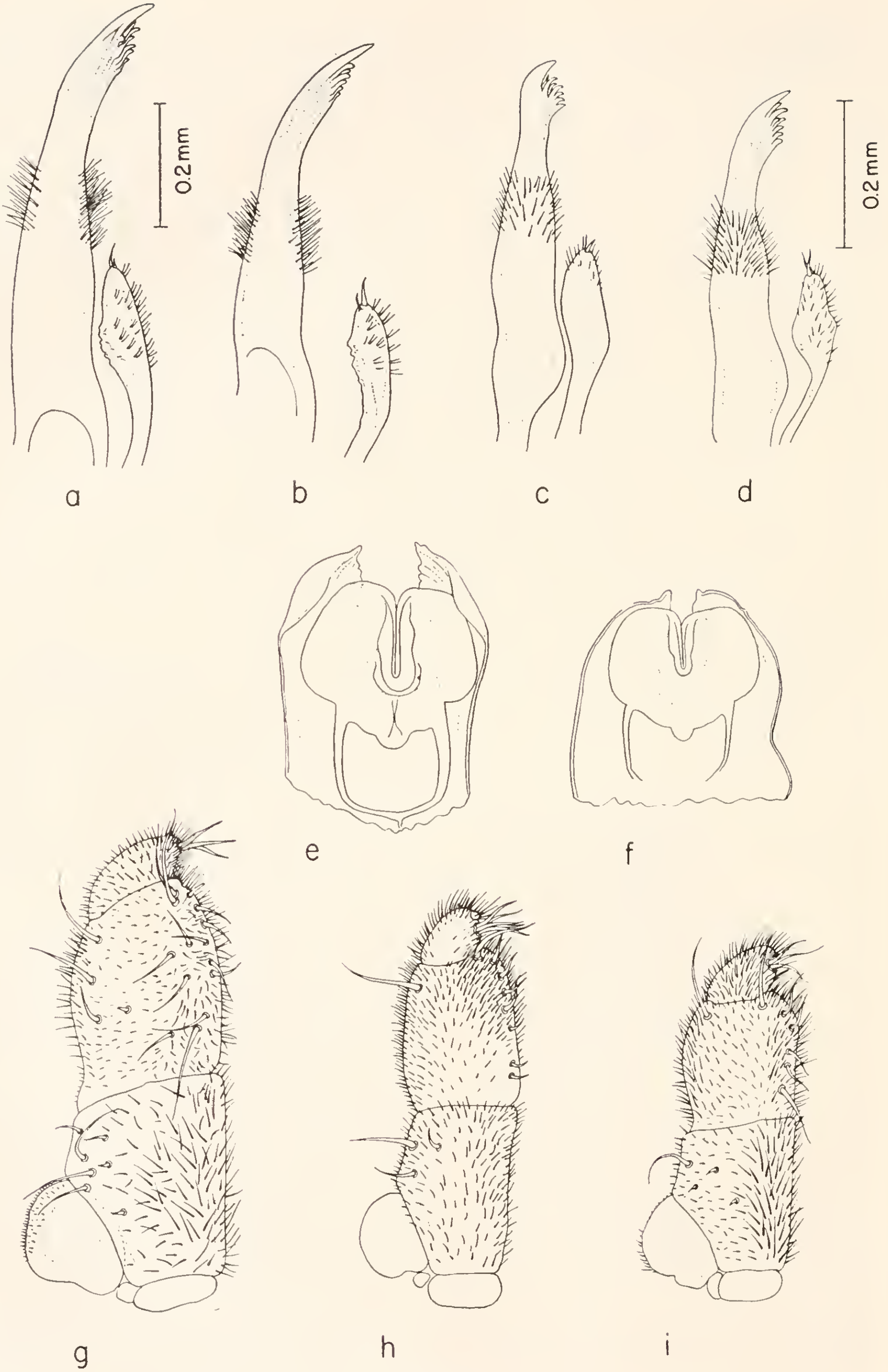
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FIGURE 4.

to its tip. The authors consider the foregoing differences in the sexual stylets of *C. polita* and *C. carinata* to be of great systematic importance.

It would be of taxonomic interest to compare the chromosomes of the two species. Burbanck and Burbanck (1958) made a preliminary report of a haploid (n) number of five for *Cyathura* sp. (= *C. polita*). Subsequent study suggests that the material on which this number was based was a stage during meiosis in the male when a larger number of chromosomes became associated into five or six groups. In addition to studying meiotic and premeiotic material, division figures have been observed in smears of developing embryos and in the somatic cells surrounding the testes. Counts of 12, about 24, and about 40 have been made, and as suitable material becomes available, further work will be done to try to establish the correct chromosome number for *C. polita*. The chromosome picture of *C. carinata* is as yet entirely unknown.

Mention has been made in the literature (*e.g.* Richardson, 1905, pp. 64–66) of intraspecific variation and some deviations have been noted in the present study. One of the variations involves the degree of fusion of the anterior five pleonites (the sixth is free). Always these are fused dorsally (a generic trait) and usually also laterally. A partial suture between the first and second pleonites is generally present ventrolaterally, however, and in some specimens three additional suture lines may be faintly indicated behind the first—a complete complement of four separating the first five pleonites. In occasional specimens, there may be only a slight indentation on the ventrolateral margin between the first and second somite (Fig. 6, i–j) with perhaps a faint line extending vertically a short distance from it. In the great majority of specimens, however, the first pleonal partial suture is quite distinct (Fig. 6, e–h). These variants are just as apt to occur in New England populations as in cyathurans in Florida. Random variation and anomalies might be expected in such vestigial structures.

Some intraspecific variation was encountered in the apex of the appendix masculinum (see Fig. 7, a–r) in both European and American species, but the essential characteristics distinguishing them were always apparent. Harger (1880, Pl. 11, Fig. f) shows the second pleopod of *Anthura polita* bearing a cylindrical stylet with a simple apex lacking any indication of the characteristic subterminal apophysis with its hooked rod (see also Richardson, 1905, p. 64, Fig. 47f). It is strange that this presumably rare variant should represent the only previous portrayal in the literature of the appendix masculinum in the American species! That Harger was aware of the typical masculine appendix is presumed from the fact that two of his slides (Nos. 188 and 191, Peabody Museum, Yale University, labelled *"Anthura brunnea"*) show the second male pleopods with the complex apex of the sexual stylet characteristic of *Cyathura polita*. The specimen he dissected was taken Aug. 28, 1874, in Noank Harbor, Connecticut. The possibility has been suggested that Harger's figure represented an immature or a "first form" male, but these ideas were largely dispelled by the observation that the lateral

FIGURE 4. Postmandibular mouthparts of American and European *Cyathura* from various regions—New Jersey (a), Mass. (b, e, g), Denmark (f, h), and England (d, i). First maxillae: *C. polita* (a, b); *C. carinata* (c, d). Hypopharynx-second maxilla complex: *C. polita* (e); *C. carinata* (f). Maxillipeds: *C. polita* (g); *C. carinata* (h, i) (d and i drawn to same scale, all others same as a).

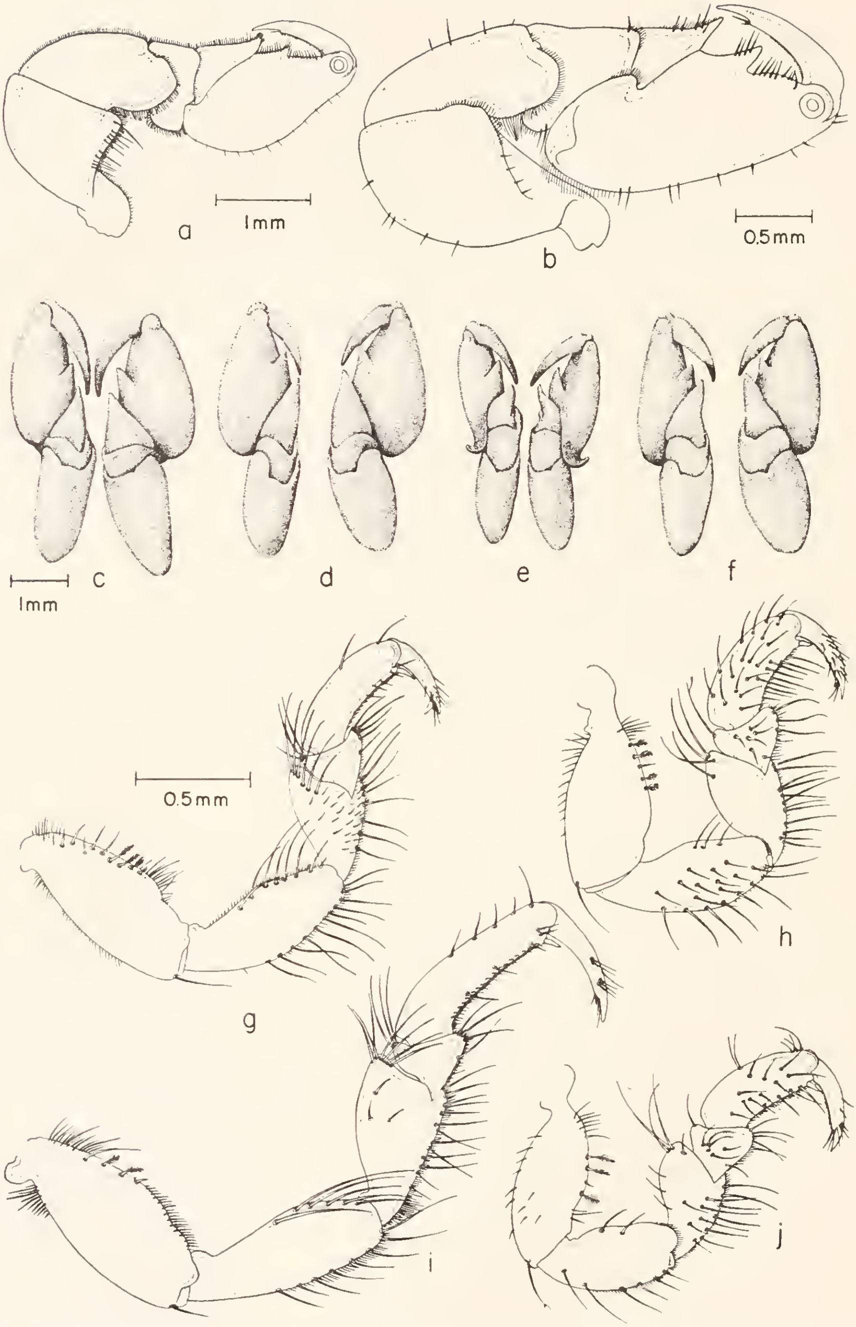

FIGURE 5. Gnathopods and sixth pereiopods of *Cyathura polita* from Stony Brook, Mass. (a), and Suwanee River, Fla. (c, d, g, i); and of *C. carinata* from Dybso Fjord, Denmark (b, e, f, h, j). Gnathopods: *C. polita*—(a) ♂, (c) ♂, (d) ♀; *C. carinata*—(b) ♂, (e) ♂, (f) ♀. Pereiopod 6: *C. polita*—(g) ♀, (i) ♂; *C. carinata*—(h) ♀, (j) ♂. Note sexual dimorphism especially in propodus of gnathopods in Danish specimens, but not in American specimens.

apophysis is indicated even before the stylet separates from the endopod (Fig. 7. u, v), and by the fact that the simple apex is apparently a rare phenomenon. The authors have found only one male of the many examined that shows an appendix comparable to that illustrated by Harger. This was from a sizable male (16 mm.) in the collection from St. Louis Bay, Miss. It was the only male in the collection. Incidentally, the pleon of the Mississippi male with the anomalous appendix showed laterally faint indications of three partial sutures behind the clear-cut first pleonal partial suture. An essentially similar condition is shown in a lateral view of the pleon by Harger (1880, Pl. 11, Fig. g). We do not know whether this structure belonged to the male with the simple appendix or if there is any correlation between the two anomalies.

A study of the dorsal chromatophore pattern of *C. polita,* with emphasis on possible geographically correlated variations, is currently being made by the junior author of this paper. Preliminary observations show that the general outlines of the pattern are the same for all the specimens of *C. polita.* but that there are variations of one part of the pattern of the first thoracic segment which are characteristic of certain geographical locations.

It is significant that many of the differences, which we observed by comparing *specimens* of *Cyathura carinata* and *C. polita,* can also be seen if one compares Schiödte's (1875, Pl. 4) figures of *C. carinata* with those given by Norman and Stebbing (1886, Pl. 27) for what they thought was the same species. Schiödte's careful illustrations were doubtless drawn from European material, whereas Norman and Stebbing admittedly based theirs on American specimens of *Anthura brunnea* (= *C. polita*) which they considered to be a synonym of *C. carinata.*

The differences in detail of the mouthparts as shown in the cited figures confirm our observations on these structures. Take the mandible for example: Schiödte shows four stout setae on the terminal joint of the palp, whereas Norman and Stebbing show 13—our counts are 4–6 for *C. carinata* and 13–14 for *C. polita.* Schiödte shows 20 serrations on the subapical flange, whereas Norman and Stebbing show 15—our counts for *C. carinata* ranged from 18 to 20, compared to 14 to 18 for *C. polita.* Or take the first maxilla: Schiödte shows the outer lamina with one large and seven small apical teeth and encircled by subapical bristles, whereas the British authors show one less apical tooth and subapical bristles only on the outer and inner margins—the same differences are shown in our figures of *C. carinata* and *C. polita* (see Fig. 4, a–d).

Besides the mouthparts, comparisons of other structures shown by Schiödte and by Norman and Stebbing indicate that these authors were dealing with distinct species. One more example will suffice: The shape of the telson, as portrayed by Schiödte, with its convex, posteriorly-converging sides is quite different from that given by Norman and Stebbing who show a strap-like telson with subparallel sides—this difference is an important distinction between *C. carinata* and *C. polita* (see Fig. 6, a–d). Unfortunately, neither of these early authors drew the appendix masculinum.

Collectively, the morphological differences detailed above, together with the zoögeographical considerations previously discussed, clinch the case for specific distinctness of *Cyathura polita* (Stimpson) and *C. carinata* (Krøyer).

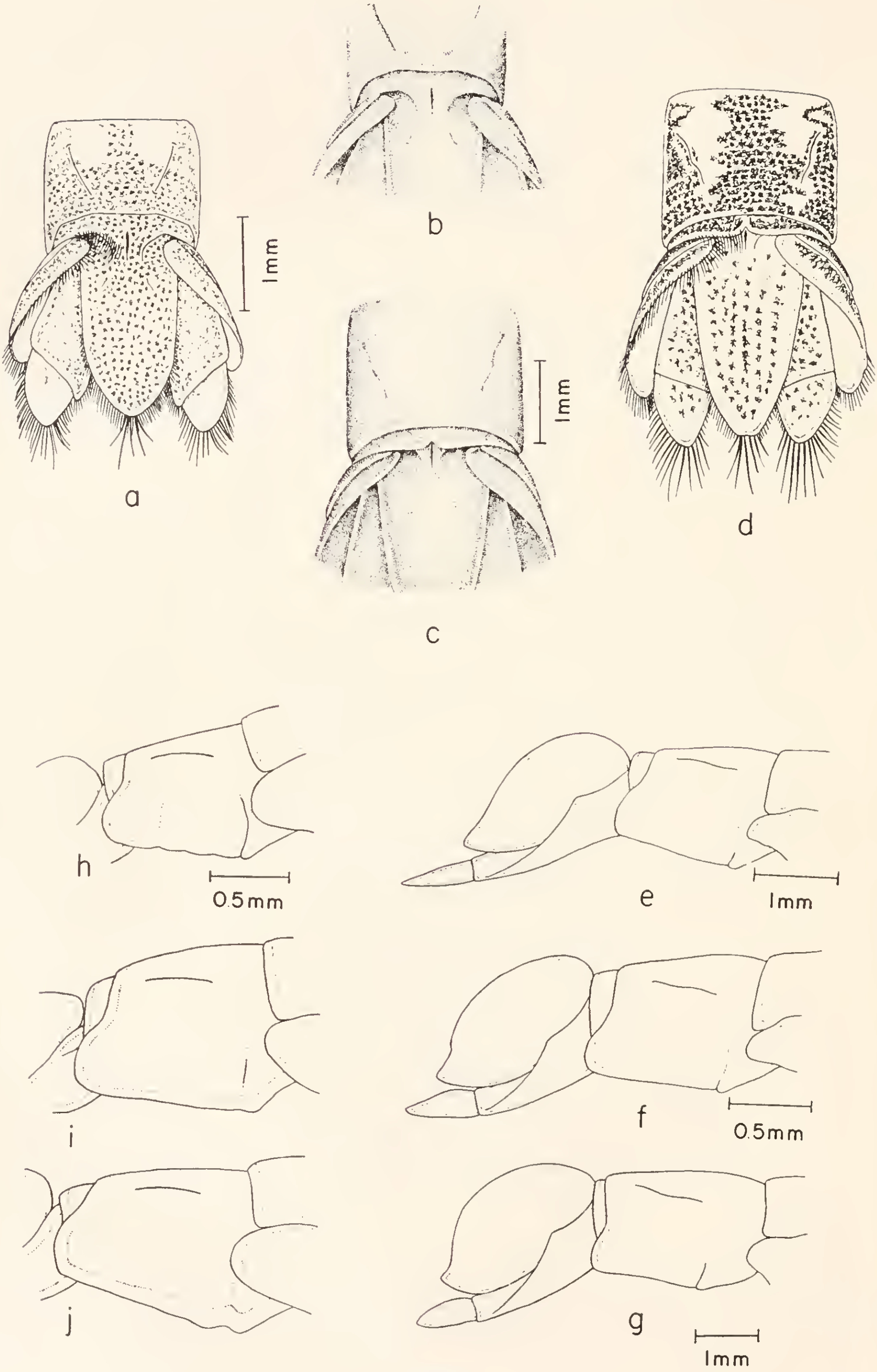
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FIGURE 6.

Localities

The geographical and ecological distributions of *Cyathura polita* along the eastern coast of the United States have recently been reported by Burbanck (1959b). The list given below comprises American localities of collections labelled *Cyathura carinata* (= *C. polita*) in the United States National Museum (USNM), locations where Burbanck (WDB) and his associates have collected this species, and certain collection sites mentioned in the literature. The list shows a long coastwise distribution of *C. polita* in lakes, bays and streams from Lake Pontchartrain, La., to Chewonki Creek, Me. Every state in this range is represented, often by several localities. On the basis of critical recheck of previous determinations, three localities (specified below) should be deleted from the distributional list of U. S. National Museum specimens given by Burbanck (1959a, p. 508).

LOUISIANA: Lake Pontchartrain (USNM 97972, also WDB).

MISSISSIPPI: St. Louis Bay (WDB).

ALABAMA: Perdido Bay (WDB).

FLORIDA: Buckhorn Creek, Florida Bay near Flamingo (R. B. Manning), Lake George, Lake Poinsett (USNM 98538), Punta Rassa (USNM 25159), St. John's River (WDB), Suwanee River (WDB). *Delete:* Frankfort Bank, Key West (USNM 68401) and Friend Key Lake (USNM 44278), as the specimens from these two localities belong to a different species of *Cyathura*.

GEORGIA: Ogeechee River (USNM 98537, also WDB), Sapelo Island (A. E. Smalley), St. Mary's River (WDB).

SOUTH CAROLINA: Ashepoo River (WDB), Cooper River (USNM 86318), Edisto River (USNM 98536), Winyah Bay (USNM 42563).

NORTH CAROLINA: Calico Creek (near Beaufort) (WDB). *Delete:* Beaufort (USNM 86338 and 86339) as these anthurids have a completely segmented pleon which eliminates them from the genus *Cyathura*.

VIRGINIA: Norfolk (Type locality, Stimpson, 1855), Potomac River (USNM 81724).

MARYLAND: Chesapeake Beach (Neotype locality), Chester River (USNM 42093).

DELAWARE: Drawyer Creek (tributary Appoquinimink Creek, one mile north of Odessa) (WDB).

NEW JERSEY: Wading River (WDB).

NEW YORK: Hudson River (at Beacon, USNM 86316; at Haverstraw, USNM 86317).

CONNECTICUT: Noank Harbor (USNM 35927).

RHODE ISLAND: East Providence (USNM 19578).

MASSACHUSETTS: Buzzard's Bay, Cape Cod and the Islands (38 localities) (WDB), Danversport (USNM 41880), Gloucester (Harger, 1880), Little

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FIGURE 6. Pleon and telson of *Cyathura polita* from Massachusetts (Centerville—a, b, h); Cider Hill Creek, Me. (e) and Salt Springs, Fla. (i, j); and of *C. carinata* from Plymouth, England (c, d, f) and Dybso Fjord, Denmark (g). Dorsal views (a–d) with setae completely or partially removed to show differences in articulation of sixth pleonite to telson. Lateral views (e–j) showing representative variations in degree of fusion of lateral partial sutures between first and second pleonites, especially noted in American specimens.

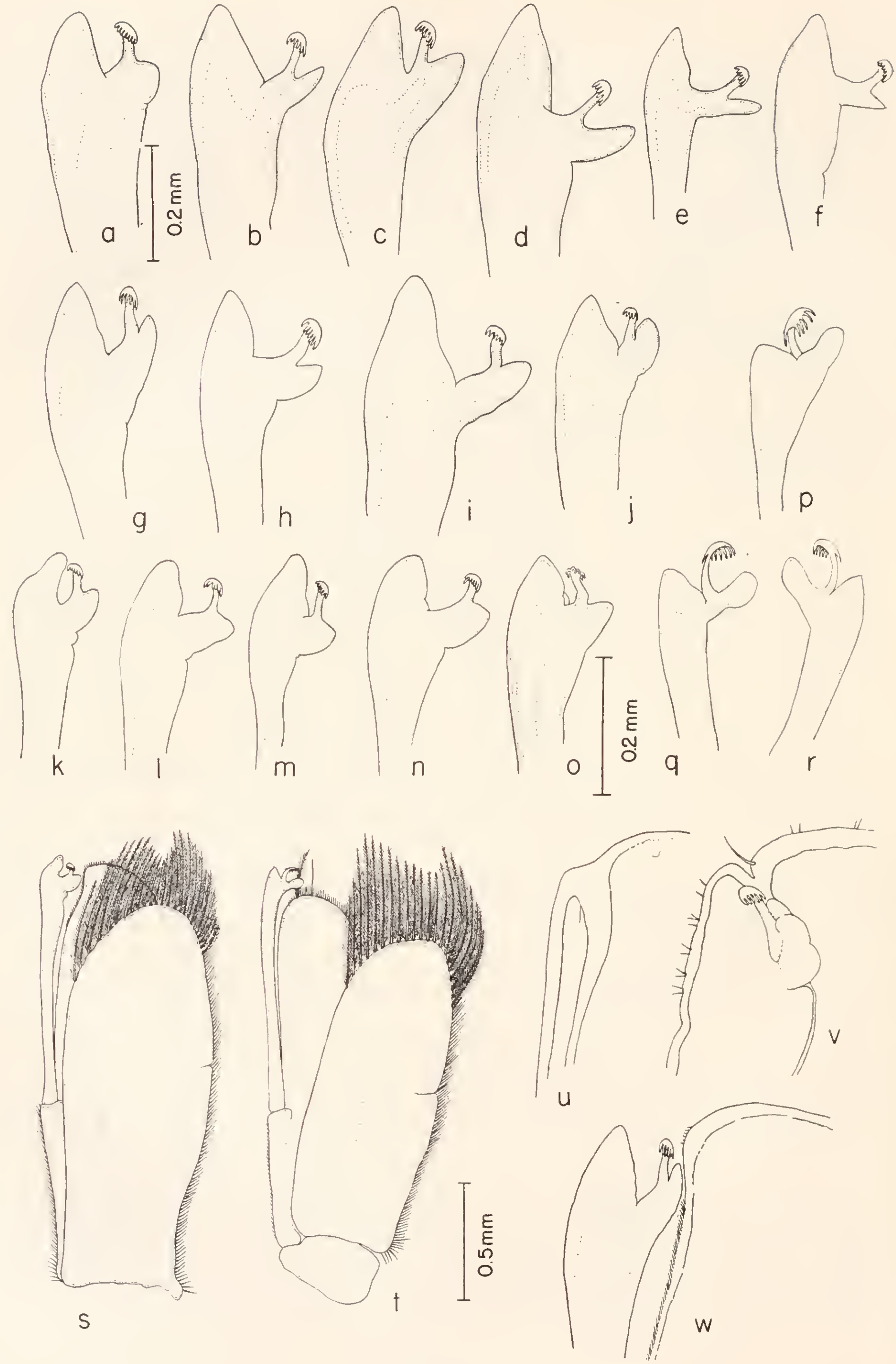
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Figure 7.

River (near West Gloucester) (WDB), North River (near Marshfield Hills) (WDB), North Scituate (WDB), Rowley River (USNM 41881), Rocky Nook (WDB), Weir River (near Nantasket Junction) (WDB), Weweantic River (WDB).

NEW HAMPSHIRE: Exeter River (WDB).

MAINE: Chewonki Creek (tributary Sheepscot River, collected by Robert Hanks and WDB), Cider Hill Creek (tributary York River) (WDB).

Type locality and types

Although Stimpson gives the type locality of *Anthura polita* as Norfolk, Va., he does not state the disposition of the type material. Recent search for the types was made at the Philadelphia Academy of Science (a logical repository) by M. A. Miller with the assistance of Dr. F. A. Aldrich and Miss Yvonne Swabey, at the U. S. National Museum by Dr. Fenner Chace, at the Museum of Comparative Zoology (Harvard) by Dr. Elisabeth Deichmann, and at Peabody Museum (Yale) by Dr. Willard Hartman. The results were negative. Dr. Hartman, however, discovered in Peabody Museum four of Harger's slides (Nos. 188–191) of various dissected parts of his *A. brunnea* (collected Aug. 28, 1874, from mud in Noank, Conn.) and one slide (No. 286) of maxillipeds and left (?) maxilla labelled "*Anthura polita* ♀" (collected May, 1878, from mud at the shore of Squan Estuary, Gloucester, Mass.). Although the mounts were in poor condition, the parts are essentially identical with the same structures from recently collected specimens. Notably, the apex of the appendix masculinum of two males from Noank Harbor showed all the specific characteristics of *C. polita.*

In view of the practical certainty that the original types are lost, it seems desirable to establish neotypes for *Cyathura polita* (Stimpson). Accordingly, we designate a collection from Chesapeake Beach, Maryland (USNM 86340, Acc. No. 160370), as the neotype series with a male as the neotype, a female as neo-allotype, and the remaining specimens as neo-paratypes. The specimens were collected July 5, 1941, by M. P. E. Morrison 100 feet off shore, 20 feet deep from sandy bottom, and identified as *Cyathura carinata* (Kr.) by J. O. Maloney. It would be desirable, of course, to select topotypes as neotypes, but since we have no specimens from the original type locality (Norfolk, Va.) we chose a reasonably close alternate. Moreover, the neotype locality is near the middle of the known range of the species. It is unfortunate that the neotype series was taken at a rather atypical depth (20 feet), but the other reasons are overriding and dictated the choice. Harger's slide material, mentioned in the preceding paragraph, might have been designated, but the authors considered that a series of entire specimens including both sexes would serve more effectively as neotypes than dissected parts of specimens taken near one extreme of the range.

FIGURE 7. Appendix masculinum and second pleopod of males of *Cyathura polita* (a–o, s, u–w) and of *C. carinata* (p–r, t). Geographical localities: Exeter River, N. H. (a); Cape Cod, Mass. (b); Red Brook, Mass. (c); Wading River, New Jersey (d); Drawyer Creek, Delaware (e, f); Silver Glen Springs, Fla. (g, h); Salt Springs, Fla. (i); Lake George, Fla. (j, v, w); Suwanee River, Fla. (k–n, s); Lake Pontchartrain, La. (o); Cider Hill Creek, Me. (u); Dybso Fjord, Denmark (p); Insel Fehmarn, Germany (q, r, t).

Ecological remarks

The conditions of existence for *Cyathura polita* have been summarized by Burbanck (1959b) as follows (p. 509):

> "1. *Cyathura* sp. live only where fresh and salt water mix.
> 2. The water covering the substrate where they live is never quiet for any appreciable length of time but is constantly in motion related to the slope of the land, tide, or wave action.
> 3. They live in simple unlined tubes of their own construction, although it is possible that they may use worm burrows as well.
> 4. Their substrate contains much or little sand with an admixture of vegetable debris, and at times, particularly in N. H. and Maine, blue clay."

He noted the high tolerance of this species both to wide and rapid changes in salinity in the laboratory as well as in the field, but observed that young were produced "far upstream," presumably in regions of low salinity.

He further stated, "With one possible exception, *Cyathura* sp. seem to live in waters of lower salinity in the south than they do in the north." The possible exception referred to was the Frankfort Bank, Florida, cyathurans which may live continuously in water having a salinity of over 30‰, unless springs of fresh water upwell in that region as they do in coastal waters off St. Augustine, Fla. With the finding that the Frankfort Bank specimens are not conspecific with *C. polita,* this exception to the quoted generalization is removed.

A unique location for *Cyathura polita* has recently been discovered by Dr. R. B. Manning, who found a colony burrowing in a marl spoilbank in Florida Bay at Flamingo, the southernmost tip of Florida. Since the spoilbank is situated near a canal that drains brackish water from Coot Bay and is exposed at low tide, it may be that the isopods are never exposed to the very high salinities (up to 70‰) reported in Florida Bay near these flats. It is thought that as the tide rises, the fresher, hence lighter, Coot Bay water would override the more saline water as it does in several estuaries in Massachusetts and Maine.

Recent collections of *C. polita* from the southern part of its range have revealed geographical differences in habitat not previously noted. From Maine to South Carolina, *Cyathura* can usually be found in that part of an estuary where there is an evident line of demarcation between the salt marsh grass, *Spartina,* and the less euryhaline cattail, *Typha.* The animals occur in waterways in a firm substrate composed of sand mixed with various combinations of gravel, clay, and vegetable debris. In both North and South Carolina, *C. polita* has been found by the junior author at approximately the upstream distributional limit of *Spartina,* but only where sand occurs where the muddy or otherwise soft bottom has been stabilized by the addition of shells, as at boat ramps or rock ballast at bridges. South of South Carolina and westward along the Gulf of Mexico, *Typha* and *Spartina* no longer consistently serve as indicators of the region where *Cyathura* may be found. Instead, *C. polita* is located in intertidal areas where there is a sandy substrate matted with roots and underground stems of tape grass, *Vallisneria,* and other littoral plants such as *Sagittaria.* Apparently *Cyathura* requires a relatively stabilized sandy substrate with the stabilizing factor varying with the latitude.

SUMMARY

A new combination, *Cyathura polita* (Stimpson, 1855), is established for a burrowing anthurid isopod widely distributed along the eastern and southern seaboard of the United States. Although it has long been considered conspecific with *C. carinata* (Krøyer), a predominantly European species, morphological comparisons, together with zoögeographical considerations, show the American forms are a distinct species first described by Stimpson as *Anthura polita*. Its known geographical range extends from Louisiana to Maine, with populations found in estuaries, tidal marshes, intertidal areas, etc., where salt and fresh water mix. It apparently requires a relatively stabilized sandy substrate with the stabilizing factor varying with the latitude.

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EFFECTS OF CULTURAL VARIATION ON CELL SIZE IN A MARINE FUNGUS [1]

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Tests for the determination of tolerance ranges of fungi isolated from salt water have indicated that some varieties show, in laboratory culture, a definite reaction to salinity and temperature, and especially to a combination of the two factors. These organisms will grow in media containing salts in about the same concentration as that found in the sea, and at temperatures they might meet in nature. When incubated at sub-optimal temperatures, they grow faster in less concentrated salts, or at supra-optimal temperatures they grow faster in more concentrated salt media.

Interdependence of salinity and temperature has been noted in a number of organisms, with a variety of criteria being used by different investigators. Johnson (1960) used infectivity of a fungus on a barnacle. Todd and Dehnel (1960) used high-temperature resistance in crabs, as McLeese (1956) did in the lobster. In all these studies, a high salinity coupled with high temperature proved favorable. An exception is the work of Broekema (1941), who, using survival time as a criterion, found that shrimp endured a low temperature better if the salinity was high.

Conclusions on the salinity-temperature relations of fungi were based, in our work, on growth rates, with growth measured as increase in colony diameter (Ritchie, 1957). We considered growth in hyphal length to be proportional to total growth of the colony, but a question arises as to whether variations in total growth of these lower forms are reflected in the sizes of individual cells. Consequently, a series of experiments, set up so as to allow for variation of both temperature and salinity, was conducted to give information on how these factors affect the cell size of a sensitive organism.

MATERIALS AND METHODS

The fungus used in the present experiments was a variety of *Phoma herbarum* West., an imperfect form isolated from yellow pine panels which had been submerged in Limon Bay, Panama. It was cultivated on agar media containing 0.5% glucose, 0.1% Difco yeast extract, and sea water which was either diluted with distilled water or concentrated by evaporation to produce a graded series containing 8, 15, 23, 30, 60, and 90 parts per thousand of salts. Cultures were prepared in triplicate, and the series at all salinities was incubated at the following temperatures: 7° C., 16° C., 25° C., 30° C., and 37° C. Figure 1 shows the results of a preliminary experiment, which indicated that at an incubation temperature of 16° C., the fungus would grow fastest in a salt concentration of about

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20 S ‰. At 25° C., the "optimum" was about 25 S ‰; at 30° C. about 38 S ‰; and at 37° C. about 48 S ‰.

Using these data as a guide, we ran two sets of cultures, one with the temperature varied as before, but with the media made up to the salt concentration which seemed optimal for each temperature, and a second set, again with the same temperatures, but with all the media made to contain 25 parts per thousand of salts. The latter concentration was chosen because, being one which will support some growth at any temperature, it would permit comparison of various temperatures with uniform salinity.

After five days of incubation, samples were cut from the colonies just back of the advancing edge of the mycelium, where the hyphae were no longer growing.

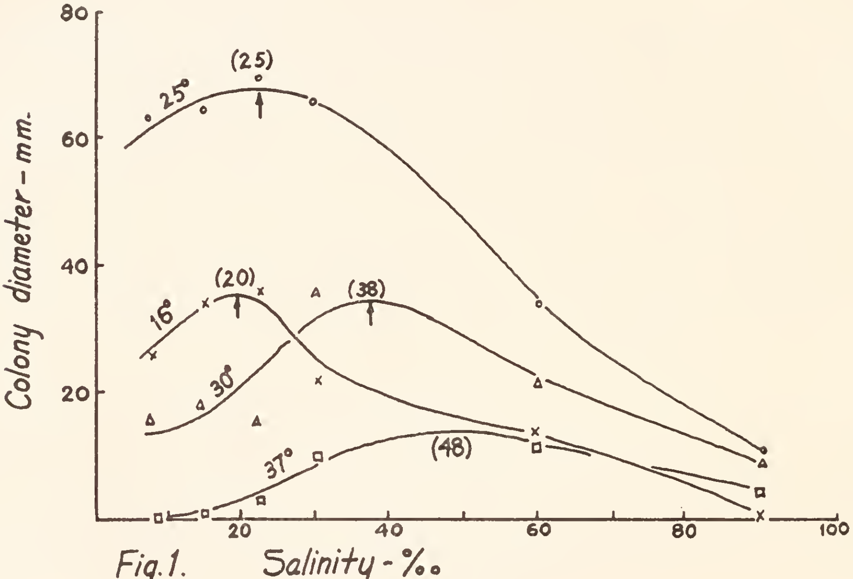

Fig. 1.

FIGURE 1. Effect of temperature and salinity on total growth of *Phoma herbarum*. The numbers in parentheses at the peak of each curve indicate the approximate salinity optimum for each temperature.

Hyphal diameters were measured with an ocular micrometer in conjunction with an oil immersion objective. Measurements were estimated to the nearest half unit of the micrometer. In any field of view, randomly picked, hyphae were measured as they fell across the micrometer scale, with subjective choice minimized as much as possible. In the experiment with salinity at 25 S ‰, fifty measurements were made on each culture; in the second, when salinity was varied, one hundred measurements were made. In one instance (25° C. and 25 S ‰), three independent, fifty-count measurements were made in order to find whether important sampling errors would show up. Inasmuch as the three runs gave consistently similar data, the number fifty was considered adequate for a sample.

Diameters only were used, and cell lengths were ignored because they are extremely variable and irregular.

The figures from any one run, as for example from 30° C. and 38 S ‰, were arranged in order, and the number of identical hyphae grouped together and counted. The resulting number was expressed as a per cent of the total count for that run, and the frequency was plotted as the ordinate, with hyphal diameters on the abscissa. Curves could thus be made comparing temperatures or salinities separately or simultaneously.

Results and Conclusions

Of the temperatures tried, the best growth of this isolant of *Phoma* occurred at 25° C. Growth at higher temperature (30° C.) or lower (16° C.) was markedly less than at the optimum, while at 37° C. it was severely retarded (Fig. 1). When cell size is considered, diameters of hyphae were, within limits, little affected by changes in temperature. Measurements from cultures incubated at 25° C. and 30° C. yielded curves that are similar (Fig. 2). Actually, the curve from the 16° C. culture is about the same, but was omitted from the graph in the interest of visual simplicity. But when the temperature was maintained at the rather high level of 37° C. with salinity kept low, the shape of the curve was altered, with a much larger percentage of thicker hyphae and a reduction in the number of slender ones. In fact, only when a definitely supra-optimal temperature was applied did the curve approach what would be expected of a normal size distribution, statistically speaking.

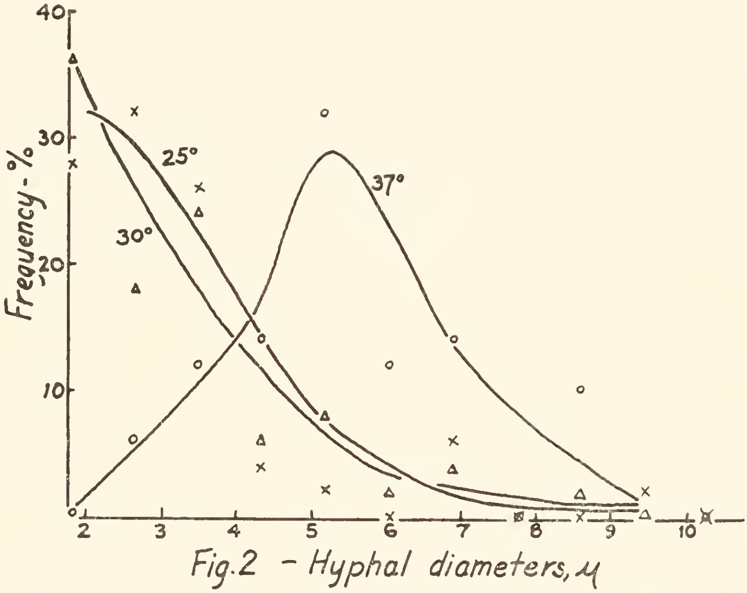

FIGURE 2. Effect of temperature on hyphal diameter. Percentage distribution of hyphal diameters in microns. Temperatures as indicated; all media containing 25 S ‰.

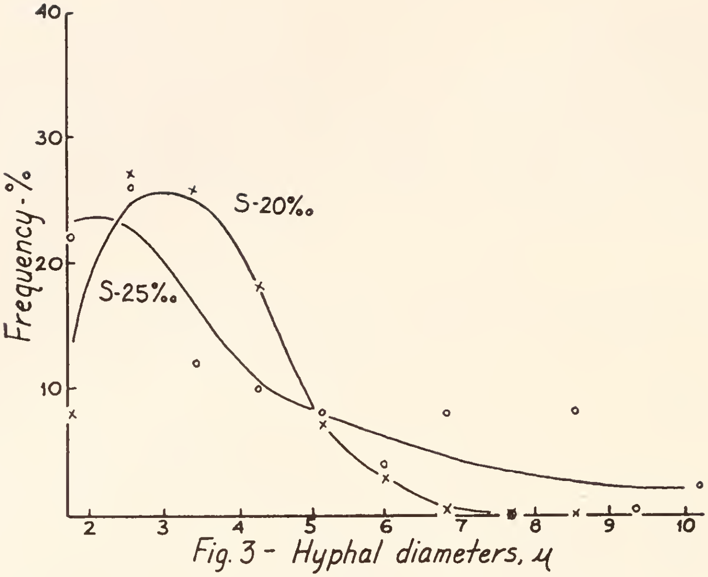

FIGURE 3. Effect of salinity on hyphal diameter. Salinities as indicated; both cultures at 16° C.

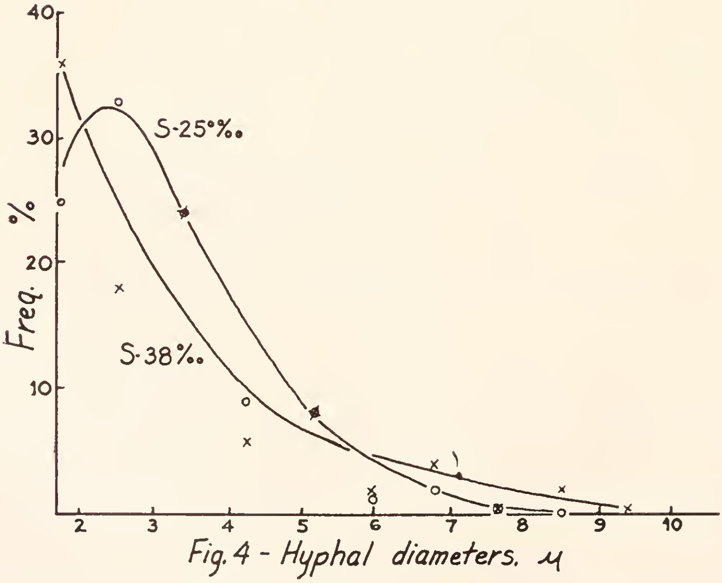

FIGURE 4. Effect of salinity on hyphal diameter. Salinities as indicated; both cultures at 30° C.

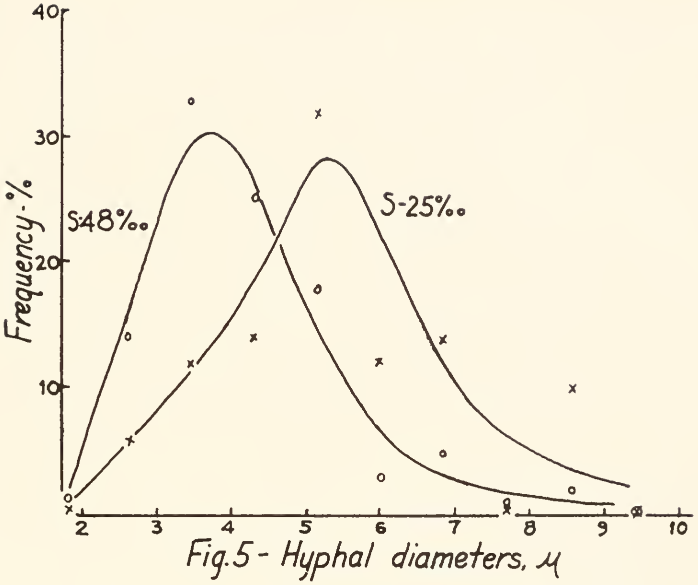

FIGURE 5. Effect of salinity on hyphal diameter. Salinities as indicated; both cultures at 37° C.

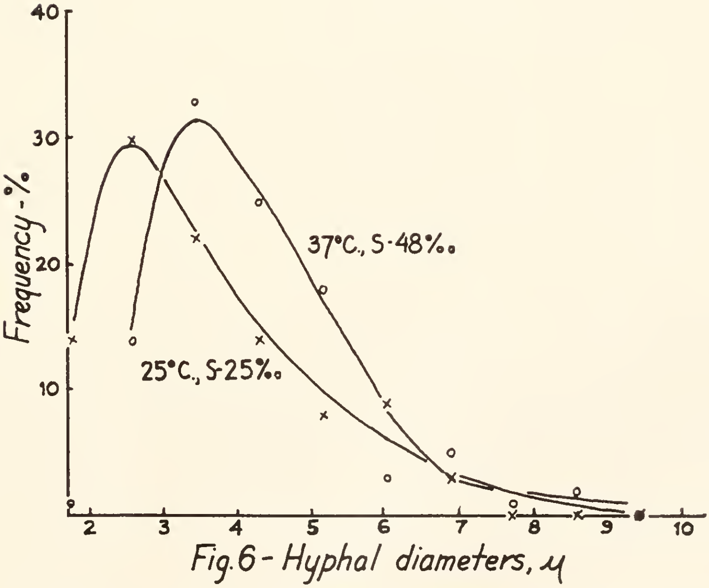

FIGURE 6. Combined effects of salinity and temperature on hyphal diameters. See text for explanation and discussion.

When salinity alone was considered, temperature being held constant, cell diameters were little altered as long as the lower temperatures were being investigated. Thus at 16° C., cultures grown at their apparent optimum for that temperature, *i.e.,* 20 S ‰, yielded data similar to those obtained from cultures from media at 25 S ‰ (Fig. 3). Essentially the same thing can be said for the cultures kept at 30° C. (Fig. 4). (Cultures at 25° C. are not treated here because the optimum at 25° C. is 25 S ‰, which happens to be the one salinity which was used for all the experimental cultures.) When, however, a run was made at 37° C., with one culture at 25 S ‰ and one at the optimal 48 S ‰, the curves show that at this high temperature the salinity of the medium made a difference in the distribution of hyphal diameters, with the lower concentration producing a more nearly "normal" curve, statistically considered, than the optimal concentration. In general, the frequency curves were strongly skewed in the direction of thicker hyphae, with relatively few of the thicker and very few of the thickest ones. Hoffman (1953) obtained similar curves, skewed to the right, in his analysis of cell size of mouse tumors. Such a distribution is not unusual in a sample like this, in which larger cells can exist, but cells below a certain minimum apparently cannot. The cultures in the higher concentration, 48 S ‰, had more narrow hyphae and fewer thick ones, in this respect resembling cultures grown at lower temperatures (Fig. 5).

This last observation is emphasized by comparison of simultaneous alteration of both temperature and salinity. When this is done, one notes a great reduction in the differences that were apparent when either factor alone was raised to a rather high level. Two curves, one from a culture from 25° C. and 25 S ‰ and one from 37° C. and 48 S ‰, are compared in Figure 6. Except for a slight preponderance of thicker hyphae in the culture from the higher temperature, the two curves are very much alike. By and large, simply raising the temperature has little effect upon the cell size of this fungus unless the temperature approaches the maximum possible, at which point cell diameters are generally increased. Similarly, raising the salinity is relatively ineffective unless the divergence in salinity is provided at a high temperature. The effect of either factor can be reduced by concomitant alteration of the other factor.

DISCUSSION

A comparison can be made between two kinds of response of the fungus to temperature and salinity variations. One is expressed as regulation of radial growth of the entire colony, and one as regulation of hyphal diameters. A similarity between the two kinds of response appears in a review of the total temperature range. As long as the colonies are incubated at low or optimal or slightly supra-optimal temperatures, the change in salinity optima with rising temperature is relatively slight, but as the temperature approaches the maximum for the organism, the salinity optimum, broad as it is, becomes much higher. Similarly, hyphal diameters are little affected by a rise in incubation temperature through the lower registers, but as the maximum is approached, the diameters are markedly greater. It should be recalled, however, that if both factors are made to approach their maxima simultaneously, cell size scarcely changes

The Coelenterate, *Cordylophora,* affords a comparable example among animals. Kinne (1958) recorded the sizes of hydranths and the number and size of tentacles in that organism, and found that when the temperature was low (10° C.), growth was greater in fresh than in saline water (30 S ‰), but at 20° C., the salinity relation reversed. This pattern in principle resembles that operating in *Phoma,* but the temperature effects upon ectodermal cell size of the animal were unlike those on the cells of the plant. Animals raised in fresh water showed a temperature effect in which the ten-degree animals had relatively wider cells than the twenty-degree ones; but animals raised in salt water (30 S ‰) showed an opposite reaction. In neither the animal nor the fungus, however, did cell size have a direct relation to total colony size, and the conclusion is evident that total size of the organism is very slightly or not at all dependent upon cell size, and that the mechanism by which temperature and salinity affect total growth is different from the mechanism by which those factors affect the size of individual cells.

SUMMARY

1. When a marine isolant of *Phoma herbarum* West. was grown in a variety of temperatures and on media containing various amounts of salts in the proportions of sea water, the size of the cells, expressed as hyphal diameters, was not greatly changed unless a temperature high enough to be inhibitory (37° C.) was applied in conjunction with a relatively low salinity (25 S ‰), under which conditions more thick hyphae and fewer thin ones grew.

2. Temperature effects upon total growth, expressed as colony diameter, and upon cell size, expressed as hyphal diameters, were similar only in that a temperature approaching the maximum is required to make any considerable change in the salinity optima or in the frequency of larger cells.

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METAMORPHOSIS OF THE VELIGER LARVAE OF NASSARIUS OBSOLETUS (GASTROPODA) IN RESPONSE TO BOTTOM SEDIMENT[1]

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Characteristic of most marine organisms is propagation of their kind by the production of great numbers of pelagic larvae. As a consequence, the dispersion of such organisms over large areas is possible. Success of benthic organisms is dependent, however, upon the metamorphosis of their larvae onto a substratum which meets their special adaptations and requirements. The metamorphosis of marine larvae upon a favorable substratum, if left to chance alone, would be indeed remote.

This hazard is apparently not as great as was once supposed, since many marine invertebrate organisms delay metamorphosis until they encounter a favorable substratum. Metamorphosis occurs as the response to a stimulus received from the desirable environment. Inasmuch as the evidence has been reviewed in a recent paper by Wilson (1952), it is necessary to relate only briefly the facts which lead to this conclusion.

The earliest laboratory observations which indicated that the substratum played a role in stimulating metamorphosis were those of Mortensen (1921) on the echinoid *Mellita sexiesperforata*. Mortensen made the casual observation that when larvae which were ready to metamorphose were placed in a culture jar with sand, metamorphosis ensued within a few days, while similar larvae from the same original culture placed in a jar without sand showed "hardly any further advance in the process of metamorphosis." This observation was later confirmed on three other species (Mortensen, 1938).

Harrington (1921) observed that shipworm larvae (*Teredo norvegica*) were positively chemotactic to a substance which he extracted from wood and which he believed was malic acid. Wilson (1952) has extended this observation and has shown that in the absence of the wood, shipworm larvae do not metamorphose. Settlement may be postponed for about one week.

Wilson (1932, 1937, 1948) and Day and Wilson (1934) observed the response of larvae of several polychaetous worms to the presence of their natural substratum. In all cases the settlement of the larvae was brought on by the addition of small quantities of substratum collected from their normal habitat. Substrata from other areas either were less effective or appeared to inhibit metamorphosis. Wilson chose *Ophelia bicornis* for a more detailed study and the results of this work have been reported in a series of papers (Wilson, 1948, 1951, 1952, 1953a, 1953b, 1954,

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[1] Contribution No. 1132, Woods Hole Oceanographic Institution.

[2] This paper is based on part of a dissertation presented to the University of North Carolina, Chapel Hill, N. C., in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

1955). While size and shape of grains seemed to have some bearing on the metamorphosis-inducing quality of the substratum, Wilson (1955) finally concluded that (p. 537) "the factor most active in inducing metamorphosis and settlement of *Ophelia bicornis* larvae is the presence on sand grains of living organisms, such as bacteria, and that these should be neither too few nor too abundant. . . . Certain species are more effective in promoting settlement than are others. . . . "

Two other species of polychaetes have shown a settlement response to the presence of substratum. Nyholm (1950) found that *Melinna cristata* (p. 88) "tended to metamorphose only in the presence of a suitable substratum rich in detritus and never when merely supplied with clear water." Smidt (1951) observed that (p. 60) "the lack of substratum will prolong larval life [of *Pygospio elegans*] while settling is furthered by the presence of a substratum." Moreover, "sterile sand and naturally occurring pebbles have an impeding effect" on metamorphosis.

In a careful study, settling of the actinotroch larvae of *Actinotrocha branchiata* [= *Phoronis mülleri*] is described by Silén (1954, p. 234): "The metamorphosis is easily and infallibly brought about by introducing some of the clay-mixed sand in which the adults are found, to a bowl containing a mature specimen." The metamorphosis which occurs "within a few usually about ten minutes" is described in great detail and involves the testing of the substratum by the larvae.

The study of Jägersten (1940) on the metamorphosis of the archiannelid *Protodrilus rubropharyngeus* is especially interesting because it describes the characteristics of a metamorphosis-inducing material. In nature *P. rubropharyngeus* has positively phototactic mature swimming larvae which, on reversing their photic response, sink to the bottom and metamorphose in a limited littoral zone with a shell or shell and pebble bottom. In the laboratory swimming larvae usually do not metamorphose but may live for two weeks without signs of transformation. If a shell or pebble from the normal habitat is included in the culture vessel, the larvae metamorphose within twenty-four hours. The material which induces this metamorphosis may be transferred to the water. It is resistant to heat, acids, and alkalis and is apparently associated with suspended material which may be removed either by centrifugation or filtration. Jägersten concluded that some non-living inorganic material is responsible for the settling response.

Wilson (1952) has concluded (p. 53 ff.): "It is evident that larvae do not settle just anywhere and that many of them have some power of selection. Over what period of time this power can be exercised is not always so clear; neither is the mechanism whereby larvae are enabled to make the selection, whether by touch, smell or other means. . . . It has proved easier to establish the facts that metamorphosis can be delayed and that larvae can choose their substratum . . . than to determine the actual features of the environment, physical, chemical or biological, perceived by the larvae and to which they react."

One of the more conspicuous organisms on intertidal flats along the Atlantic coast from the Gulf of St. Lawrence to northern Florida is *Nassarius obsoletus* Say. This prosobranch gastropod is extremely abundant in its preferred habitat. Newly metamorphosed snails, during favorable years, occur in large aggregations which reach densities of 23,000 individuals per square meter. A patch of newly metamorphosed snails, while usually not uniform in density, may exceed 100 square meters in area. Apparently success of the species is due to the settlement of the larvae in a favorable environment; an explanation may be that the larvae metamorphose in response to some clue provided by the environment.

The experiments which are described in the following pages are offered in support of such a hypothesis. They show that in the laboratory, the larvae of the gastropod mollusk *N. obsoletus* are able to recognize a favorable substratum and can, to a considerable degree, delay metamorphosis until a suitable substratum is come upon. Hence the "power of selection" in choosing a place for settlement is proven to exist in yet another group of invertebrate animals. The experiments also suggest the singular manner by which the larvae achieve this end.

I should like to acknowledge with gratitude the financial assistance afforded through a fellowship from the Woods Hole Oceanographic Institution during the summers of 1956, 1957, and 1958, and the encouragement offered by Dr. Bostwick H. Ketchum of that institution.

Mr. Harry J. Turner has been most generous in making available all the facilities of his laboratory. At various times during the course of the research he offered comments and suggestions which were helpful and for which I am indebted.

I am grateful to Dr. Charles E. Jenner for his suggestions and advice at certain critical points of the research and for his careful reading of the manuscript.

With Amelie, my wife, I have shared some exciting moments of discovery, but also many evenings counting over larvae and editing the manuscript.

EXPERIMENTAL PROCEDURES

The experiments that have demonstrated the relationship between the substratum and metamorphosis in *Nassarius obsoletus* have been simple in their design. Inasmuch as all of these experiments involved quite similar methods, the general procedure used will be described here; the details are presented later together with the results.

All experiments entailed the use of at least one control and one experimental container, each with twenty veliger larvae. The containers, which were of translucent polyethylene,[3] were filled with one-half liter of sea water that had been filtered through glass or dacron wool.

The bottom sediment to be tested was placed in the experimental dishes. About 5 cc. of substrate material were required to just cover the bottom of a container. The substratum, unless otherwise indicated, was screened before use by passing it through either a Number 60 (240 μ) or 100 (140 μ) stainless steel screen, or a Number 2 (316 μ) bolting cloth. This removed all organisms larger than *ca.* 0.3 mm, which interfered with the conduct of the experiment. The substratum usually was composed of a very fine sand and included a considerable amount of organic material.

The controls consisted of containers with filtered sea water only; in some cases, when the substratum was treated in a manner to be explained later, additional controls with untreated substratum were added to the series.

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[3] Manufactured by the Kordite Corporation, Macedon, N. Y. Polyethylene to which coloring matter has been added is unsuitable, presumably because of the toxic effect of the dye.

Control and experimental containers were started simultaneously with larvae grown in and picked at random from the same culture. The larvae at the beginning of the experiment were at a stage of development which precedes metamorphosis, *viz.,* the creeping-swimming stage, attained about 3 weeks after emergence from the egg capsule. Subsequently both experimental and control containers were placed together on a sea table or nearby shelf. Hence the larvae are presumed to have been maintained under similar but not necessarily constant or uniform conditions throughout the course of their growth previous to the experiments and also, except for the conditions being tested, throughout the duration of the experiments. Temperature throughout the experiments ranged from 22° to 24° C.

Most experiments were terminated after 24 hours; some ran only 12 hours and others lasted up to four days.The larvae or recently metamorphosed young were removed from the plastic containers either by pipetting them directly from the containers or by carefully screening the contents of the containers so as to permit the substratum to pass through, leaving the organisms behind. The number of larvae that had metamorphosed was then determined by inspecting each larva under a dissecting miscroscope. The criterion used to determine metamorphosis was the loss of the velum.

A concise description of the method used in rearing the larvae will suffice, inasmuch as a more complete account will be given elsewhere, together with the external morphology of the larvae. Egg capsules were obtained in the field or from snails held in the laboratory. After emergence from the egg capsules the larvae were collected on a fine mesh screen, placed into 15-liter rearing vessels, and aerated. Water in the rearing vessels was changed every second or third day by passing the entire contents of the vessel through a fine mesh screen which retained the larvae. *Phaeodactylum tricornutum* Bohlin (Ehr.) W. Sm. at a concentration of 2×10^5 cells/ml. was used as a source of food. This diatom may be readily grown in mass culture.

A brief explanation of the experimental data given in the tables is required. Since the age of the larvae, as well as previous cultural conditions, was of necessity not identical from one group of experiments to the next, any set of experiments performed at one time are not strictly identical to those of another. The past history of the culture and the difference in the age of the creeping-swimming larvae explain much of the variability observed in the data. It is clearly valid, however, to compare the results from a series of experimental containers with those derived from their respective controls. The statistical significance of the difference between such means has been determined by the Student t-test. The percentages metamorphosed were corrected for mortality, and all statistical computations are based on the number of living organisms recovered at the end of the experiments.

REACTION OF NASSARIUS OBSOLETUS TO THE SUBSTRATUM

a. *Metamorphosis in the presence of natural substratum*

The importance of the substratum to onset of metamorphosis in *Nassarius obsoletus* is readily demonstrated. In the presence of a bottom sediment on which

TABLE I

Percentage metamorphosis of Nassarius obsoletus *in the presence of of natural substratum (results from ninety-four experiments)*

Experimental condition	Per cent mortality	Mean per cent metamorphosed*
Natural substratum	2.1	70.0 ± 2.4
Without substratum	1.4	18.9 ± 1.4

* One standard error is indicated on all values throughout this paper.

juvenile *N. obsoletus* are found in nature, the percentage metamorphosis is very significantly greater than in controls without such natural substratum. The results of 94 experiments show that with a natural substratum 70.0% of the larvae ended their planktonic existence, whereas in controls of filtered sea water only, 18.9% of the larvae metamorphosed. The value of P in these experiments equals less than 0.001. Moreover, in not one of the above experiments did the percentage of metamorphosis in the control ever exceed that in the experimental culture. The results of the experiments are summarized in Table I. The larvae at the end of

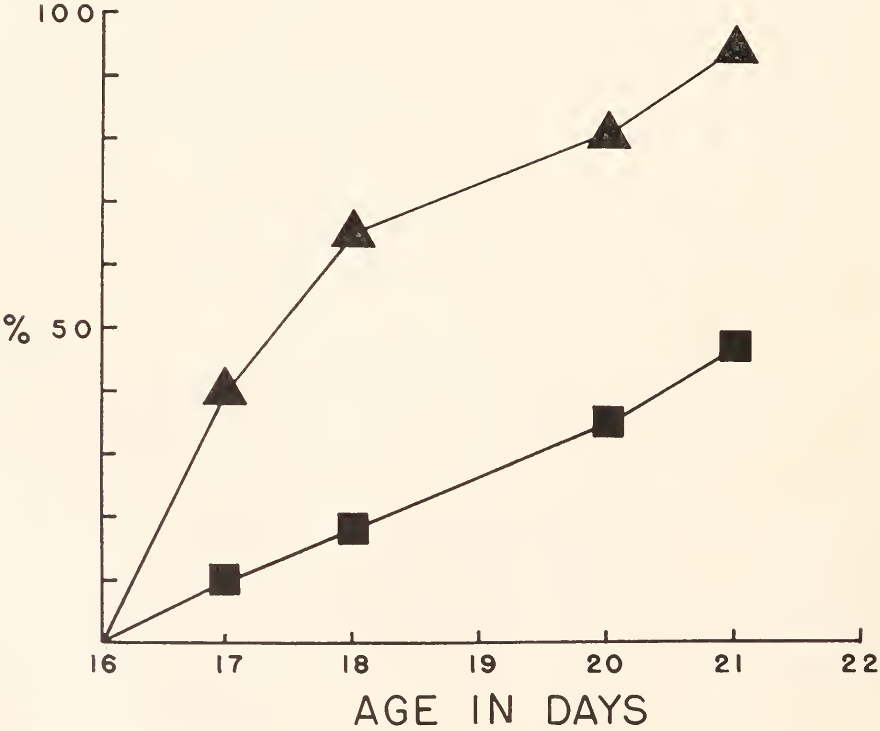

FIGURE 1. Percentage metamorphosis with (▲) and without (■) natural substratum in *Nassarius obsoletus.* Fifty larvae, 16 days after emergence from egg capsules, were placed in each of two 2-liter containers. In the bottom of one was placed freshly collected substratum; no substratum was placed in the control. The percentage metamorphosis in each container is shown at the times indicated on the abscissa.

the experiments ranged in age from 16 to 35 days (mean 25.7) after emergence from the capsule. The eggs were obtained from snail populations in the vicinities of Woods Hole, Massachusetts, and Beaufort, North Carolina.

Apparently metamorphosis cannot be indefinitely delayed. In the absence of bottom sediment the tendency to metamorphose is a simple linear function of age (Fig. 1). The maximum delay which was demonstrated was 20 days after the creeping-swimming stage had been attained. In the presence of bottom sediment the tendency to metamorphose is also a function of age; however, this tendency increases very rapidly and is not a simple linear function. In a series of experiments (Scheltema, 1956), it was shown that when 20-day-old larvae were used, 17% metamorphosed in response to bottom sediment, whereas in similar experiments using 31-day-old larvae 90% metamorphosis resulted.

TABLE II

Mechanical analyses of substrata from three areas used in metamorphosis experiments*

Mesh no.	Openings in mm.	Per cent passing through sieve		
		Substratum #1	Substratum #2	Substratum #3
5	4.000	82.8	—	—
10	2.000	75.4	—	—
18	1.000	57.5	100.0	100.0
35	0.500	24.3	99.6	97.7
60	0.250	6.3	94.4	92.0
120	0.125	0.5	66.2	84.1
230	0.062	—	7.3	77.3
Median diameter		0.9 mm.	0.1 mm.	<0.06 mm.

* Substratum #1: Head of Great Pond (off mouth of Perch Pond), East Falmouth, Cape Cod, Massachusetts (see Hulburt, 1956). "Favorable" substratum in preference experiments.

Substratum #2: Experimental area of Woods Hole Oceanographic Institution, Barnstable Harbor, Cape Cod, Massachusetts (see Ayers, 1959).

Substratum #3: Mouth of Great Pond (west shore), East Falmouth, Cape Cod, Massachusetts (see Hulburt, 1956). "Unfavorable" substratum in preference experiments.

b. *Preference for a favorable substratum*

Certain natural substrata are apparently preferred over others, inasmuch as a significant difference in the response of *Nassarius obsoletus* larvae to different substrata may be demonstrated experimentally. A sustratum sample obtained from a location shown to be very favorable for metamorphosis of *N. obsoletus* (#1, Table II), as indicated by the large number of snails observed to have metamorphosed there in the previous two years, was compared with another sample collected from a region believed to be unfavorable for metamorphosis (#3, Table II), as demonstrated by the complete absence of recently metamorphosed snails during the same two-year period. The latter region, although unfavorable for metamorphosis, was not inimical to the existence of the species, since older snails of three years or over regularly were found to inhabit this flat. Plankton tows revealed great numbers of larvae during the months of July in the waters over both regions from which the substratum samples were obtained.

The two substrata differed markedly. That taken from the area where juvenile snails were abundant was composed largely of coarse sand and fine gravel. It was poorly sorted, with a median grain size of 0.9 mm. An abundance of living diatoms was present in this substratum. The other substratum was rich black and probably somewhat anaerobic since a trace of hydrogen sulfide odor could be detected. This material was less than 0.06 mm. in median diameter and may be characterized as silt. Viewed under the microscope it appeared to contain large amounts of organic detritus. The mechanical analyses of the substrata are summarized in Table II. The substrata used in the experiments were not screened, but all larger organisms were removed from them with forceps under a dissecting microscope.

TABLE III

The preference of Nassarius obsoletus *for a favorable substratum for metamorphosis (results from seven experiments)*

Experimental condition	Per cent mortality	Mean per cent metamorphosed
Favorable substratum (#1, Table II)	0.0	84.4 ± 8.3
Unfavorable substratum (#3, Table II)	0.0	51.6 ± 7.6
Without substratum	0.0	30.2 ± 11.0

The percentage metamorphosis on the two substrata may be compared in Table III. In the coarse substratum 84.4% of the larvae metamorphosed, whereas on the fine black organic substratum 51.6% ended their planktonic existence. This difference is statistically significant (P equals less than 0.025). The difference between the fine black substratum and the control without substratum was not significant (P equals 0.20). The mean age of the larvae at the beginning of the experiment was 25.0 days.

The experiments show that substrata favored in the laboratory are similar to those preferred under natural conditions, and that there is a correspondence between experimental results and field observations. The general application of these results should be viewed with considerable reservation, however, pending further experiments using other favorable and unfavorable bottom sediments.

PROPERTIES OF THE SUBSTRATUM WHICH INDUCE METAMORPHOSIS

Numerous experiments indicate that the induction of metamorphosis by a favorable substratum is not a direct function of its physical character, but rather the result of its biological and chemical properties. Two lines of evidence support this conclusion.

a. *Physical properties of the substratum*

During the course of many experiments, sediments have been used from widely diverse regions. Mean grain size and sorting have differed greatly. Of those substrata that have been tested experimentally and proven effective in inducing metamorphosis, the greatest extremes in physical properties are to be found in substrata #1 and #2 in Table II. The former, from the head of Great Pond, East Falmouth, Massachusetts, is poorly sorted gravel and sand. The latter, from Barnstable Harbor, Cape Cod, Massachusetts, is well sorted and consists primarily of fine reworked glacial sand. For the purpose of the experiments, the Barnstable Harbor substratum was passed through a Number 2 (316 μ) bolting cloth. This sieving had little effect on the physical character of the sediment, removing at most perhaps 2% of the total sample, *i.e.*, that portion exceeding 0.3 mm., in diameter. The Great Pond sample was not screened, but mollusks and annelids were removed. Table IV shows the results obtained with these substrata: in the Great Pond sample 84.4% of the larvae metamorphosed, while in the Barnstable substrate 88.7% completed their planktonic existence. Although experiments with the Barnstable and Great Pond sediments were conducted at different times and with larvae from different cultures, it seems justifiable in this instance to compare results and to conclude that the wide difference in physical character of the substrata had no effect on their ability to influence metamorphosis. The experimental results are completely in accord with the observations in nature that the larvae in fact do metamorphose in large numbers on these two substrata.

TABLE IV

The effect of two substrata, differing widely in physical properties, on the percentage metamorphosis of Nassarius obsoletus

Substratum used	Character of substratum	No. of expts.	Mean per cent metamorphosed with substratum	Mean per cent metamorphosed without substratum
Great Pond, Falmouth, Mass. #1, Table II	0.9 mm. median diameter, poorly sorted sand and gravel	7	84.4 ± 8.3	30.2 ± 11.0
Barnstable Harbor, Mass. #2, Table II	0.1 mm. median diameter, well sorted fine sand	18	88.7 ± 3.3	21.0 ± 4.8

It may be concluded from this evidence that, within wide limits, mean particle size and sorting have no direct effect on metamorphosis. There are, however, certain indirect effects of particle size on the biological properties of the substratum as a consequence of decreased surface with increasing particle size. ZoBell (1946) has shown, for example, that the size of bacteria populations may be related to particle size.

b. *Biological and chemical properties of the substratum*

Response of larvae to the biological and chemical attributes of the substratum was tested in a series of experiments. It was intended to alter the sediment biologically and chemically without changing its physical properties.

Treating a substratum sample by (1) incinerating, (2) oven-heating, (3) washing, (4) heating in a water bath, or (5) irradiating with ultraviolet light results in each case in a significant reduction of the metamorphosis-inducing properties of the substratum.

Incineration was accomplished by first washing and boiling the sediment in distilled water several times to remove most of the organic material, and then placing the cleaned sediment in a crucible and heating it above the high flame of a bunsen burner for one hour. The result of this treatment was to render the substratum abiotic and to free it of all traces of organic matter.

Oven-heating the sediment was done at 210° C. for 15 to 30 hours. The dried sample when removed from the oven had a dark brown appearance and a slightly pungent odor. This substratum also was abiotic, but differed from the incinerated substratum in probably having some organic material still present.

Washing the sediment consisted of boiling it in sea water and then in distilled water for several minutes, stirring vigorously, and finally decanting the surplus fine organic material. This process was repeated until the sample appeared essentially clean, *i.e.,* until it assumed a more or less light gray color and lacked any apparent suspended material when stirred. This treatment resulted in removal of part of the organic material and perhaps much of the natural microflora.

Heating the sediment was done by placing a small flask containing some sea water and the sediment sample into a beaker of water and raising the temperature to 70° to 90° C. for five to fifteen minutes. When the experiments were performed, the sea water heated inside the flask with the substratum was used along with new sea water added to make up a volume of one-half liter. Hence no part of the original substratum could have been lost, even if some material is assumed to have gone into solution as a result of the treatment. It was hoped that as a result of this treatment most of the micro-organisms would be killed without substantially affecting the organic material in the sediment. Actually this manipulation resulted, after a period of 24 hours, in a greater total number of micro-organisms than found in the natural substratum, presumably owing to the increased growth of saprophytic bacteria on dead micro-benthos. The difference amounted to two orders of magnitude as determined by plate counts, using ZoBell's marine medium No. 2216 (ZoBell, 1946). The resulting micro-flora was also qualitatively different. The treatment did not appear to cause anaerobic conditions and no fouling of water occurred.

Irradiation with ultra-violet light was done by placing, under a 15-watt germicidal lamp, containers with sediment covered by sea water to a depth of 1½ cm. The treatment was continued 18 hours, the containers being occasionally agitated. After treatment additional sea water was added to each container. Preliminary studies showed that UV treatment reduced the number of bacteria culturable in a marine medium (ZoBell's 2216) by two orders of magnitude if plated after two hours of treatment. The results of the UV treatment were not entirely successful in that complete sterilization is not possible. There is a minimum bacterial count below which further treatment is ineffective even with frequent agitation. Moreover, subsequent to irradiation, growth of saprophytic bacteria is probably very great because of the increase in decomposable material (dead micro-benthos).

The five treatments, as is shown in Table V, all resulted in a significant reduction in the attractiveness of the substrata, as determined by their effectiveness in promoting metamorphosis. Comparison of the experimental groups with their

TABLE V

The effect of various treatments on the metamorphosis-inducing property of the substratum

Treatment of substratum	No. of expts.	Mean age of larvae at start	Mean per cent metamorphosis in treated substratum	Mean per cent metamorphosis in control (untreated substratum)	Difference between treated substratum and control
Incineration	14	24.6	7.2 ± 1.5	71.2 ± 5.3	64.0
Oven-heating (210° C.)	11	26.8	19 0 ± 4.5	69.7 ± 5.0	50.7
Washing	10	22.8	4.6 ± 1.2	51.5 ± 3.9	46.9
Heating in water bath (70°–90° C.)	24	28.4	40.0 ± 4.7	81.2 ± 3.8	41.2
UV irradiation (18 hrs.)	5	28.0	70.4 ± 6.7	91.4 ± 3.4	21.0

untreated controls gives values of *P* less than 0.001 in all cases except that of the UV irradiation, in which a value of 0.05 was obtained.

Inasmuch as all the treatments shown in Table V have in common the fact that they drastically affected the biological properties and to varying degrees the chemical properties of the substrata, without greatly affecting the physical properties, one may conclude that biological and chemical attributes of the substrata are responsible for their metamorphosis-inducing property.

The various treatments of the substrata in these experiments (Table V) cannot be directly compared with one another because the experiments were conducted with different samples of sediment and with larvae of different ages from different cultures. Nevertheless, the differences between the treated and the control substrata (right-hand column) give some suggestion as to the relative effectiveness of each treatment in destroying the metamorphosis-inducing property of the substrata.

Further experiments have confirmed that a statistically significant difference does indeed exist between the various treatments of the substrata and their attractiveness in inducing metamorphosis. The experiments shown in Table VI were performed with larvae from the same culture and with substratum from a single source. Their only differences were the experimental conditions indicated in the left-hand column. Heating of the substratum in a water bath to 90° C. for

TABLE VI

Relative effect of two treatments of the substratum on its metamorphosis-inducing property (results from fourteen experiments)

Experimental condition	Per cent mortality	Mean per cent metamorphosed
Untreated substratum	1.5	71.2 ± 5.3
Substratum heated in water bath to 90° C.	3.4	33.3 ± 4.8
Incinerated substratum	0.0	7.2 ± 1.5
Without substratum	1.6	13.9 ± 2.6

15 minutes rendered it very significantly less attractive than the normal substratum; however, it also remained very significantly more attractive than incinerated substratum or the controls without substratum (*P* equals less than 0.001 in both instances). No significant difference resulted between incinerated substratum and the controls without substratum (*P* equals approximately 0.10).

These experiments show that the treatment of heating the substratum in a water bath at 90° C. yielded results which fall approximately midway between those using the natural substratum and the incinerated substratum.

The experiments on the effects of various treatments of the substratum indicated that: (1) complete removal of the micro-flora and -fauna and of all organic material rendered the substratum unsuitable for stimulating metamorphosis of veliger larvae; (2) qualitative and quantitative changes in the bacterial flora, accompanied by (a) the removal of living microscopic algae and micro-fauna and (b) the alterations in the organic material, owing to heat and addition of decomposition products from the micro-benthos, made the substratum less attractive in inducing metamorphosis; (3) the biological and possibly the chemical character of the substratum was the determining factor in making the substratum suitable for metamorphosis.

A micro-benthic community cannot be separated from the organic material in a sediment. Therefore there is no experimental procedure by which to measure the effect of a micro-benthic community as a whole upon the metamorphosis-inducing property of the substratum. Single species isolated from the substratum may be tested if members of the micro-benthos can be isolated. Preliminary experiments on the effects of benthic marine bacteria isolated from favorable substrata have not yielded consistent results. The effect of the organic material on the suitability of the substratum theoretically may be tested, but no satisfactory method is available to sterilize a substratum without altering the organic material present.

NATURE OF THE METAMORPHOSIS RESPONSE: EXPERIMENTAL EVIDENCE FOR THE PRESENCE OF A SOLUBLE, BIOLOGICALLY ACTIVE, SUBSTRATUM FACTOR

How do the larvae perceive a favorable substratum? Is actual contact with the substratum necessary or can the larvae distinguish a favorable place for metamorphosis by some sensory clue received from material liberated into the water? Is some biologically active substance or perhaps ectocrine (see Lucas, 1955) responsible for the response to the substratum? The experiments which follow were designed to answer these questions.

a. *Transfer of the metamorphosis-inducing factor from the substratum to adjacent water*

When sea water is permitted to stand over a substratum favorable for inducing metamorphosis of veliger larvae, it soon acquires some of the metamorphosis-inducing property. Evidently some of the qualities of the substratum are transferred to the sea water.

The water was conditioned by filling a large fingerbowl to 2 cm. in depth with a sediment favorable for metamorphosis. The remainder of the bowl was then filled with fresh, filtered sea water and allowed to stand in a south window for several days to a week. Carefully the sea water was then siphoned off, particular care being taken to exclude particles of the sediment. Filtered sea water not treated with substratum was used as control. A total of ten different substratum samples was used. In some cases, because of the time involved in treatment, the conditioned sea water was re-used. The results of the experiments are shown in Table VII.

The mean percentage metamorphosis in the substrate-conditioned sea water was 48.5 and differed significantly from the untreated control in which only 11.2% of the larvae metamorphosed (*P* equals less than 0.001).

The results showed that a metamorphosis-inducing factor could be transferred from the substratum into the water to a significant degree, and that larvae were able to discriminate between water recently exposed to a favorable substratum and water not recently in contact with such a substratum.

Table VII

The effect of sea water conditioned with substratum on the percentage metamorphosis of Nassarius obsoletus *(results from twenty-four experiments)*

Experimental condition	Per cent mortality	Mean per cent metamorphosed
Substrate-conditioned sea water	1.1	48.5 ± 4.9
Untreated sea water control	1.2	11.2 ± 2.5

b. *Filterability of the substrate factor through membrane filters*

The metamorphosis-inducing substrate factor, if soluble, should pass through a molecular filter unaltered. An experiment was performed similar to those just described except that the substrate-conditioned sea water was divided into two equal parts. One half was filtered through a molecular filter[4] with a pore size of 0.8 μ; the other half was retained as a control. Both water samples were then tested for their metamorphosis-inducing property against a sample of untreated sea water. The resulting metamorphosis of larvae in the unfiltered, substrate-conditioned sea water and the filtered, substrate-conditioned sea water is shown in Table VIII. The mean age of the larvae at the beginning of the experiments was 28.8 days. Twelve and three-tenths per cent of the larvae in the untreated, 46.9% of the larvae in the substrate-conditioned, and 33.0% of the larvae in the filtered, substrate-conditioned sea water metamorphosed within a period of 48 hours. The difference in results between the filtered, substrate-conditioned water and the untreated sea water was significantly different (*P* equal to less than 0.001). The difference between the filtered, substrate-conditioned sea water and unfiltered, substrate-conditioned sea water was not significant since the value of *P* was approximately 0.10.

The experiments showed that the metamorphosis-inducing property of the substratum was at least in part a water-soluble substance; previous evidence has already indicated that it must be relatively stable at temperatures likely to be encountered in nature. One may speculate that the metamorphosis-inducing factor can be adsorbed by the sediment since experiments using the substratum showed a consistently higher per cent metamorphosis over those with substrate-conditioned sea water. Such a comparison is not valid, however, without further experiment using similar larvae and sediments. The difference between the mean percentage metamorphosis in the filtered and unfiltered substratum-conditioned sea water, though not statistically significant, may perhaps indicate that under certain circumstances some adsorption may occur either on fine suspended or colloidal particles, which are removed on filtration, or on the filter itself.

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[4] Millipore Filter Corporation, Watertown, Massachusetts.

c. *Implications of the experiments using substrate-conditioned sea water*

The two series of experiments using substrate-conditioned sea water supply the evidence which makes possible a reasonable hypothesis to explain the nature of the response to the substratum. The experiments suggest that the metamorphosis response of *Nassarius obsoletus* larvae is due to a soluble substance, which is relatively stable, and of biological origin. Such a substance may justifiably be termed a "biologically active" substance. Apparently the larvae perceive this substance without contact with the substratum. The latter conclusion presupposes that the veligers must have chemoreceptors sensitive enough to discriminate between the presence or absence of the metamorphosis-inducing factor in the water.

TABLE VIII

The effect of filtrating the metamorphosis-inducing property of the substratum through a molecular filter (results from eighteen experiments)

Experimental condition	Per cent mortality	Mean per cent metamorphosed
Substrate-conditioned sea water	1.2	46.9 ± 6.5
Molecular filtered substrate-conditioned sea water (0.8 μ pore diameter)	0.3	33.0 ± 5.6
Untreated sea water	0.3	12.3 ± 3.0

Observations on adult *N. obsoletus* under both laboratory and field conditions indicate a high degree of chemoreception. For example, snails placed in a tank with a small piece of clam meat are able to locate such food very rapidly by testing water through their siphons. Chemical sensitivity has been demonstrated on two European species of the same genus (Henschel, 1932; Weber, 1924). In view of these well-developed chemical sense organs observed in the adults of the genus, it is not too surprising to encounter a well-developed chemoreception in the creeping-swimming stage of *N. obsoletus.*

Two types of chemoreception may be distinguished (Carthy, 1958): a contact chemical sense and an olfactory sense. The distinction is largely that of the concentration required to stimulate. Inasmuch as contact with the substratum by *N. obsoletus* is unnecessary, olfaction, the more sensitive chemical sense, is probably involved in the initial response.

Once the creeping-swimming larva descends to the bottom, further testing of the substratum is possible by contact chemoreceptors. This may provide an explanation for the relatively higher percentage of metamorphosis in those experiments using a substratum over those in which substrate-conditioned water was used.

Biologically active substances encompass a wide variety of materials and have been demonstrated to be of importance in a great diversity of biological activities. The subject has recently been extensively reviewed by Collier (1953) and Lucas (1955). We shall therefore not attempt an extensive summary here. It will suffice to say that the regulation of many marine animal relationships, including commensalism, antibiosis, and predation, have been explained by the demonstration of a biologically active substance.

A large diversity of organic compounds are apparently to be found in the ocean. Collier in 1953 reviewed the types of organic substances which had been found up to that time. Among these he listed N-ethylcarbazol substances ("carbohydrates"), tryosine, tryptophane, rhamnoside, ascorbic acid, and certain other unknown materials, among these a yellow lipoid substance. To these may now be added many other materials which have recently been discovered such as B_{12}-active substances (Burkholder and Burkholder, 1956; Starr *et al.,* 1957), organic acids (Koyama and Thompson, 1959; Slowey *et al.,* 1959), and various algal (Proctor, 1957) and bacterial (Sieburth, 1959) antibiotics.

Clearly, biologically active substances must encompass a wide variety of materials. Collier (1953) believes that organic compounds may originate (1) as degradation products of dead or dying animals and plants, (2) as excretory products, (3) as diffusible metabolites, and (4) from microbial activity. In the case of the metamorphosis response of *Nassarius obsoletus* to bottom sediment we do not yet know the chemical identity nor the exact source of the biologically active material. "A mud or soil with an abundant and varied microflora should contain very nearly the gamut of microbial metabolites . . ." (Hutner and Provasoli, 1951). Moreover, it is not apparent whether more than one substance may be involved in the response of the larvae. The evidence shows no discrete threshold at which metamorphosis occurs, inasmuch as the response of a group of experimental larvae to the metamorphosis-inducing factor is not "all or none." Hence not all creeping-swimming larvae metamorphose after 24 hours, despite the presence of a suitable substratum. Obviously the metamorphosis response to the substratum is not a simple one, nor should simplicity in behavior be expected from larvae, inasmuch as they are in most anatomical and physiological respects fully as complex as adults.

FUNCTION AND ECOLOGICAL SIGNIFICANCE OF THE SUBSTRATUM IN INDUCING METAMORPHOSIS IN NASSARIUS OBSOLETUS AND NASSARIUS VIBEX

a. *Adaptive value of the response of larvae to the substratum*

Nassarius obsoletus, which ingests large quantities of bottom material by means of its proboscis, is a deposit feeder. The evidence for this comes from the examination of fecal material and digestive tract contents. Since a crystalline style is present (Jenner, 1956), it is presumed that large amounts of carbohydrates are digested from the microflora within the sediment. Living bivalves of any size or species which have been tried in the laboratory are consistently refused, including those which are frequently associated with *N. obsoletus* (*e.g.,* recently metamorphosed *Venus mercenaria* and juvenile *Gemma gemma*). Subsistence from dead mollusks or Crustacea is possible; indeed, on intertidal flats one frequently encounters aggregations of several hundred *N. obsoletus* feeding on some dead marine organism. Such dead organisms cannot, however, form a principal item of diet because of the large size of the *N. obsoletus* populations found on the intertidal flats.

Observations on another species of the genus *Nassarius* suggest that there is a correspondence between the metamorphosis response to bottom sediment and the adaptive value of such a response. *Nassarius vibex,* which is sometimes found sympatrically with *N. obsoletus,* is a scavenger. It is found much less abundantly than *N. obsoletus,* as would be expected by its trophic position in the community in which it lives. Experimentally we have shown that *N. vibex* does not metamorphose in response to a bottom sediment. A series of eleven experiments resulted in 67.4 ± 11.8% metamorphosis when sediment was present on the bottom of the experimental containers, whereas 62.9 ± 11.5% metamorphosed without the presence of sediment. Hence the complex behavior pattern of *N. obsoletus,* which results in the metamorphosis of larvae on a favorable substratum, has considerable adaptive value; such a response seemingly would be of no marked advantage to *N. vibex.*

b. *Ecological significance of the response of* Nassarius obsoletus *to the substratum*

"The continued presence of an animal in an environment depends upon the developmental stage in which it has the least adaptability and this stage, the weakest link of the chain, will normally be found during the breeding period and larval development." (Thorson, 1957, p. 472.) Hence, less stability should be expected of populations of organisms which have long pelagic larval stages. A comparison of arctic bottom communities, where almost all benthic organisms have direct development, with tropical benthic communities, where 75 to 80% of all bottom forms have pelagic larvae, displays no marked difference in the stability of the two (Thorson, 1957). There must exist a means, aside from chance, by which tropical benthic communities maintain their relative stability. Indeed the stability of any bottom community which depends largely on recruitment of pelagic larvae needs explanation.

One interpretation of the relative stability of large benthic populations may be found in the response of larvae to a metamorphosis-inducing factor in the substratum or, more accurately, to a very complex behavior pattern which consists of (1) recognition of a favorable substratum or environment and (2) a response to this favorable substratum, terminating in metamorphosis. This type of response may be expected in benthic forms which are deposit feeders. Our experiments have shown that under optimum laboratory conditions larvae of *N. obsoletus* may metamorphose as early as 17 days after their emergence from the egg capsule, while in the absence of a favorable substratum the termination of planktonic existence may be delayed for as long as 20 days after the creeping-swimming stage has been attained. Although the favorable substratum is come upon accidentally, the larvae may delay their metamorphosis over a period of sufficient time so as to make such an encounter very highly probable. Estuaries, which form the chief habitat for *N. obsoletus,* often have circulations of the type which will carry swimming larvae over long distances upstream along the bottom (Pritchard, 1951). Larvae as they are carried by bottom currents over a particularly suitable region will metamorphose.

Summary

1. Experimental evidence shows that the presence of a favorable substratum has a marked effect on the metamorphosis of *Nassarius obsoletus* larvae. In the presence of a natural substratum the percentage metamorphosis was very significantly greater than in controls without such natural substratum. Larvae postponed metamorphosis for over two weeks when a desirable substratum was not encountered.

2. Certain substrata were preferred over others; a significant difference in the response of *N. obsoletus* larvae to different sediments was demonstrated experimentally. A correspondence exists between substrata preferred under natural conditions and those favored in experiments in the laboratory.

3. The physical properties of a substratum, such as median grain size and sorting, do not directly influence the metamorphosis of *N. obsoletus* veliger larvae. This was demonstrated both experimentally and by field observations.

4. Certain biological properties are important in making the substratum attractive for the metamorphosis of *N. obsoletus* veligers. This was shown by the differences in settlement obtained experimentally when the sediment was treated in various ways. When a sediment was rendered completely abiotic by incineration, no significant difference existed between the percentage metamorphosis on such a substratum and in controls without substratum. If the biological characteristics of the sediment were drastically altered by heating in sea water, the attractiveness of the substratum to metamorphosis by veliger larvae was significantly reduced, but it remained nevertheless significantly more attractive for metamorphosis than a substratum which had been made completely abiotic.

5. The metamorphosis-inducing properties of a substratum may be transferred to the adjacent water, as was demonstrated experimentally.

6. Experimental evidence showed that the metamorphosis-inducing factor from the substratum is probably a water-soluble substance.

7. Inasmuch as the response to a metamorphosis-inducing factor is possible without contact with the substratum, a rather sensitive chemoreception by *N. obsoletus* larvae must be involved. Stimulation may be followed by a complex behavior pattern which includes further testing of the substratum and subsequent metamorphosis.

8 Since *N. obsoletus* is primarily a deposit feeder, the selection of a favorable substratum has considerable adaptive value.

9. The ability to delay metamorphosis and the response to a favorable substratum greatly increase the probability that the larvae of *N. obsoletus* will terminate their pelagic existence in a favorable habitat.

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A PERFUSION STUDY OF THE MOVEMENT OF STRONTIUM ACROSS THE GILLS OF RAINBOW TROUT (SALMO GAIRDNERII)

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Strontium uptake by fresh-water and marine fishes has been previously studied by either feeding, immersing, or otherwise exposing the intact fish to the isotope, and counting the activity gained by the animal. These methods give an understanding of the gross over-all uptake and elimination of the isotope, but offer little or no comprehension of the function of the gills or the mechanisms involved.

Preliminary feeding and immersion studies in our laboratory indicated that the gills of the rainbow trout were a major pathway for the uptake of strontium from water, although strontium was not concentrated in the fish to any great degree. In addition, the gills of fish are known to have an important role in homeostasis by their ability to actively take up some ions and to excrete metabolic products. Therefore, it was decided to investigate the rates of influx and outflux of strontium across the gill membrane as a first step in the study of the metabolic pathways and regulation of incorporated strontium in fish.

Keys' (1931) basic method for the perfusion of the gills of the eel was adapted for rainbow trout. The results obtained by this method suggest outflux rates compatible with the relatively poor ability of fish to concentrate strontium in the body from the water or food.

MATERIALS AND METHODS

A diagram of the gill perfusion preparation is presented in Figure 1. The internal system consisted of a perfusion pump to force a modified Ringer's solution through the gills, out the dorsal aorta, and into a reservoir. The pump simultaneously drew the fluid from the reservoir into a scintillation detector from which it was recirculated through the gills. The scintillation detection system consisted of a well-type crystal which contained a coil of small-bore glass tubing through which the perfusate flowed. The output of the detector was fed to a pulse height analyzer focused on the .513 mev peak of Sr^{85}. The signal from the analyzer was integrated by a precision count rate meter and recorded on a Sanborn four-channel recorder.

In addition to isotopic concentration, the flow, volume, and pressure of the internal system were recorded electronically. Flow and volume were recorded as hydrostatic pressure in the reservoir (Fig. 1, g). Efferent flow from the gills returned to this reservoir via an automatic syphon (Fig. 1, f) which emptied when

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[1] Based on work performed under Contract No. AT(45-1)-1350 for the U. S. Atomic Energy Commission.

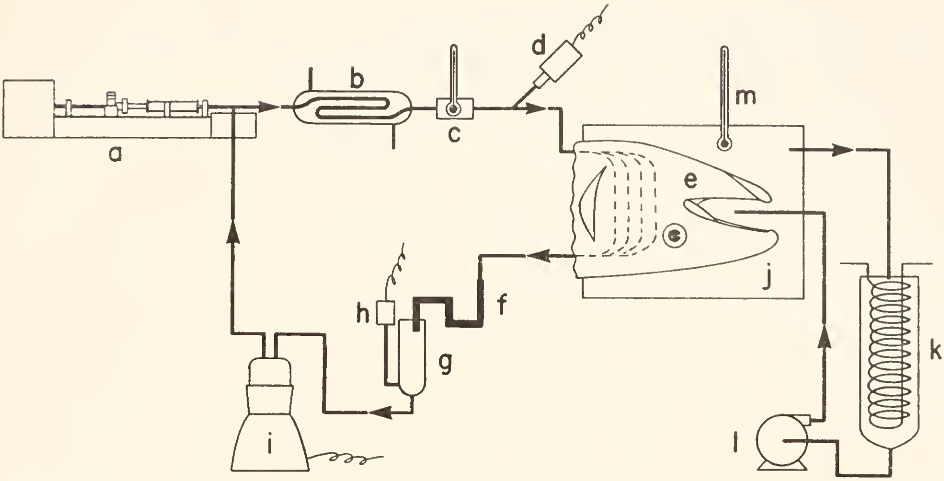

FIGURE 1. Diagram of gill perfusion preparation. Internal system: (a) perfusion pump, (b) cooling coil, (c) thermometer, (d) electronic pressure transducer, (e) fish, (f) automatic syphon, (g) reservoir with sidearm and pressure transducer (h) to measure volume and flow, (i) scintillation detector. External system: (j) diaphragm tank, (k) reservoir with cooling coil, (l) pump, (m) thermometer.

it attained its full capacity of 2.8 ml. This addition to the reservoir caused a measurable increase in hydrostatic pressure, and by use of the time scale on the electrogram, flow could be calculated. By the same means, an over-all decrease in hydrostatic pressure could be calibrated as a loss of volume in the internal system. Internal system pressure was measured as mm. Hg by a standard Sanborn medical pressure transducer (Fig. 1, d).

The Ringer's solution used had the following composition:

5.0 mEq K^+/l.
6.5 mEq Ca^{++}/l.
109 mEq Na^+/l.
112 mEq Cl^-/l.

This particular balance, which contains more Ca^{++} than frog Ringer's solution, had been found to support a trout's heart satisfactorily. Just before use, 0.4 g. $NaHCO_3$ and 0.9 g. glucose per liter of stock were added for buffer and nutrient.

The external system consisted of two liters of fluid which was pumped through the buccal and opercular cavities in order to maintain a good flow over the gills. Both the internal and external solutions were buffered in a like manner and aerated with 95% oxygen + 5% carbon dioxide gas so that the pH of the fluids remained at 6.5 to 6.7 throughout all experiments. Temperature was controlled in both systems at 13° C., an optimum for rainbow trout.

The fish was anesthetized in a solution of 0.2 g./liter of MS-222 (Tricaine Methanesulfonate, Sandoz) until respiratory movements ceased. After being weighed, the fish was placed in a holder and a median ventral incision was made from just anterior to the anus to about one centimeter posterior to the insertion of the pectoral fins. Then heparin was injected into the heart and the gastrointestinal tract, liver, gall bladder, gonads, and air sac were removed. All main arteries, the air bladder, and the esophagus were ligated.

A cross-sectional incision was made in the mid-kidney to open the dorsal aorta, and the heart was fully exposed. The ventricle was separated from the atria and opened to receive a polyethylene cannula. The tip of the cannula was positioned in the bulbus arteriosus and tied. The time period from the start of the operation to this point was no longer than 6 or 7 minutes.

Once the gills were being perfused with the Ringer's solution, the dorsal aorta was cannulated by exposing the aorta at the site of the earlier incision in the kidney and inserting a snug-fitting polyethylene tube until its tip lay well in the region of the head kidney. Successful positioning was obtained by palpation of the kidney as the cannula was inserted. Once positioned, slight vacuum was applied to the open end to establish a good flow. This end of the cannula was kept about 5 cm. below the level of the fish, to avoid extreme pressure in the gills. Due to vascular resistance in the healthy gills a pressure of 45 to 75 mm. Hg was present in the afferent section of the system. Additional afferent pressure gained by the raising of the tip of the cannula will destroy the preparation.

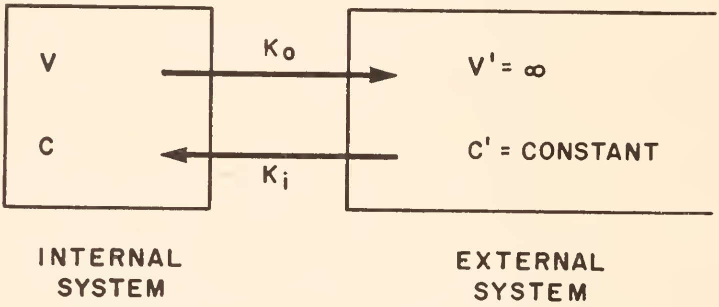

FIGURE 2. Model of two-compartment system as used in gill perfusion studies. C and C′ = concentration, V and V′ = volume.

Once the afferent and efferent flows were well established, the coelom was filled with a thick solution of gelatin just warm enough to be fluid. This solidified rapidly and sealed all bleeders. The fish's head was then inserted through the rubber diaphragm into the tank; the external system was filled with an appropriate fluid and circulation begun.

After the preparation had been completed and the blood flushed out of the gills, the flow from the efferent cannula was directed into the flow meter and recirculation was started.

The basic model of the preparation (Fig. 2) shows V′, the external volume, to be essentially infinite and C′, the external concentration, to be constant, due to the comparatively large volume of the external system and the short period of data collection. Actual measurements of concentrations in this system before and after experiments proved these assumptions to be valid.

Errors caused by volume changes due to osmotic forces or leakage from the internal system were difficult to evaluate. Leakage through the gills was found to be the most serious. By minimizing the time period during which data were collected it was felt that these errors would have no serious reflection on the basic conclusions.

Keys (1951) fully discussed the difficulties encountered in this type of experimentation.

RESULTS

Data acquired by this experimental method, using strontium-85 as a tracer ion, were considered as a linear regression of the internal concentration against time. Although the extended curve from transfer data of this type would be expected to be exponential, it was discovered that the linear treatment would introduce little error at this point and eliminate much of the difficulty encountered in endeavoring to handle both the influx and outflux with the same mathematical approach.

The equations

$$\frac{C_t \times V}{t \times A \times C'_o} = I \qquad (1)$$

and

$$\frac{C_t \times V}{t \times A \times C_o} = O \qquad (2)$$

were applied to the data for calculation of the influx rate (I) and outflux rate (O). C_t is the internal concentration at time t. C'_o and C_o are the initial external and internal concentrations, respectively. V is the initial internal volume, and A is the area of the gills. The area of the gills in relation to body weight was estimated from Price's (1931) figure of 2.2 cm.^{2} of gill area/kg. of body weight, which was determined on the black bass, *Micropterus dolomieu*. Although species differences probably exist, the error is constant throughout this study. I and O, the flux rates, have the dimensions of velocity, *i.e.*, cm./sec., and are similar to the permeability constant (Davson, 1951).

TABLE I

Influx rate (I) for strontium transport through the gill of rainbow trout

Exp. no.	Nutrient Ringer's solution internal concentration at t = o (μg. Sr^{++}/ml.)	Buffered distilled water external concentration at t = o (μg. Sr^{++}/ml.)	I (cm./sec.)
1	0	Sr^{85} (trace carrier)	1.1×10^{-8}
2	0	Sr^{85} (trace carrier)	1.3×10^{-8}
3	0	Sr^{85} (trace carrier)	2.6×10^{-8}
4	0	Sr^{85} (trace carrier)	1.0×10^{-8}
10	0	10 μg./ml.	0.5×10^{-8}
15	0	1 μg./ml.	5.2×10^{-8}
16	0	1 μg./ml.	12.0×10^{-8}
17	0	Sr^{85} (trace carrier)	7.7×10^{-8}
20	0	Sr^{85} (trace carrier)	15.0×10^{-8}

Mean ± Standard Error $5.2 \times 10^{-8} \pm 1.8 \times 10^{-8}$

TABLE II

Outflux rate (O) for strontium through the gill of rainbow trout

Exp. no.	Nutrient Ringer's solution internal concentration at t = o ($\mu g.\ Sr^{++}/ml.$)	Buffered distilled water external concentration at t = 0 ($\mu g.\ Sr^{++}/ml.$)	O (cm./sec.)
7	100 $\mu g./ml.$	0	1.1×10^{-5}
8	10 $\mu g./ml.$	0	1.1×10^{-5}
12	1 $\mu g./ml.$	0	1.8×10^{-5}
13	1 $\mu g./ml.$	0	1.3×10^{-5}
14	1 $\mu g./ml.$	0	1.4×10^{-5}
Mean ± Standard Error		$1.3 \times 10^{-5} \pm 0.1 \times 10^{-5}$	

In order to illustrate the treatment of the data received from this method, a protocol of a typical influx and a typical outflux is given. The results determined in similar experiments are listed in Tables I and II.

Determination of the influx rate

Experiment 1

Fish weight: 1094 g.

Estimated gill area: 2400 cm.^{2}

Sex: Male

Internal volume: 130 ml.

Internal medium: Nutrient Ringer's solution

External medium: Buffered distilled water plus trace amount of $Sr^{85}Cl_2$. This gave 625,000 cpm. (C′) in the scintillation detector.

Figure 3 shows the data from this experiment. Internal pressure, flow rate, and volume loss were used as physiological parameters of the condition of the gills. Extreme deviations in any one of these parameters would cause the experiment to be discarded.

By using the data from Experiment 1 in Equation (1) the value of I can be calculated:

$$\frac{130\ \text{cpm./cm.}^3 \times 130\ \text{cm.}^3}{2100\ \text{sec.} \times 2400\ \text{cm.}^2 \times 312{,}000\ \text{cpm./cm.}^3} = 1.1 \times 10^{-8}$$

cpm./cm.^{2}/sec./external concentration, or 1.1×10^{-8} cm./sec.

Table I shows the values of I determined in nine similar experiments. It can readily be seen that I appears to be independent of the initial concentration of the external system. Although the flow rate through the gills may or may not affect the rates developed here, every effort was made to keep this constant and all experiments were run at flow rates of 3.5 to 4.5 ml./min.

Determination of the outflux rate

Experiment 7

Fish weight: 1598 g.

Estimated gill area: 3500 cm.^{2}

Sex: Female

Internal volume: 80 ml.

Internal medium: Nutrient Ringer's solution plus 100 μg. Sr^{++}/ml. labeled with Sr^{85}. When circulated through the detector a count rate of 72,000 cpm. was obtained, corresponding to 360 cpm./μg. Sr^{++}.

External medium: Glass-distilled water buffered with 0.4 g. $NaHCO_3$/l.

The change in the concentration of Sr^{++} in the internal medium was estimated from the count rate resulting from the Sr^{85}, and is shown in Figure 4.

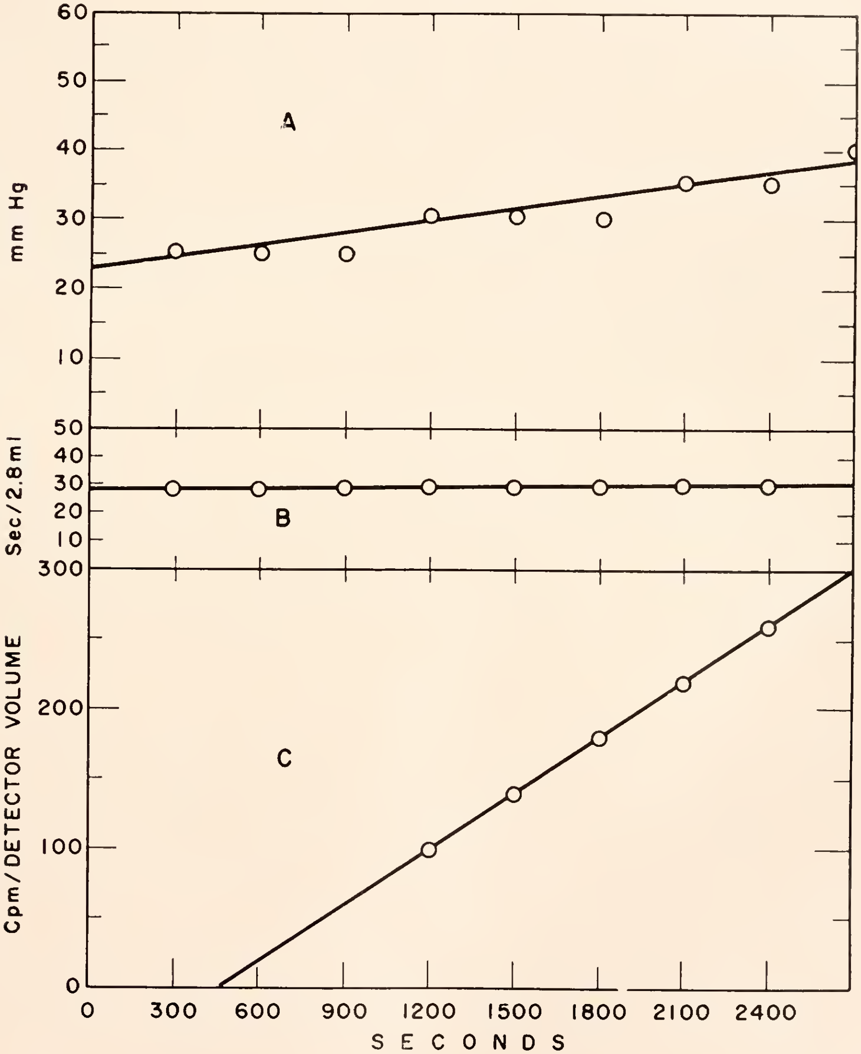

FIGURE 3. Data taken from original electrogram recorded during Experiment 1. (A) Internal pressure in mm. Hg; (B) flow in sec./2.8 ml.; (C) count rate due to Sr^{85} in cpm./detector volume. Points are discrete measurements from a continuous recording.

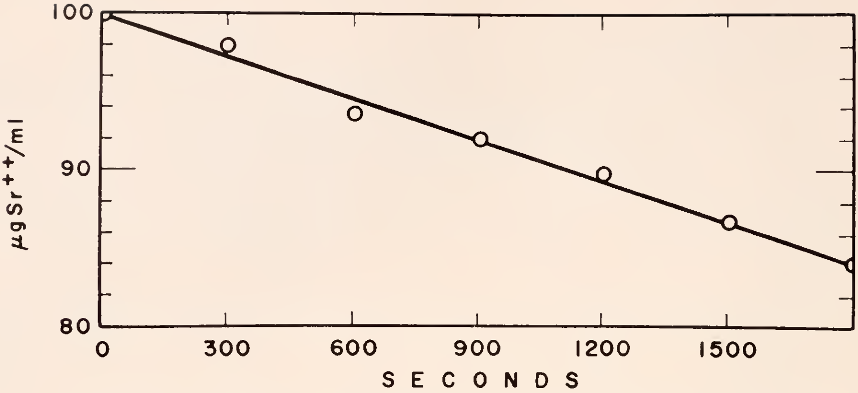

FIGURE 4. Internal concentration during course of Experiment 7.

In order to illustrate the fact that "C" can also be expressed in μg. Sr^{++}/ml., and to give some indication of the amount in μg. crossing the gill, the data from this experiment were expressed in μg. rather than in counts per minute.

The outflux rate can be calculated by Equation (2). The outflux rate for experiment 7 was 1.1×10^{-5} cm./sec.

Table II shows the O values determined in five similar experiments. Like I, O appears to be independent of the initial concentration.

DISCUSSION

Since the variance of O is greater than the variance of I, and the number of samples were unequal, Cockran's modification of Behrens-Fisher t test (Snedecor, 1956) was used and showed a marked difference between O and I at the 5% level.

If the rate at which the ion is transported through the membrane, as expressed by the flux rate, times the concentration of the ion, represents the amount that is transported through an area per unit time, then at equilibrium:

$$I\,C' = O\,C \tag{3}$$

It can readily be seen from the values of I and O obtained in this study that the concentration in the internal system at equilibrium will only be about 4% of the external concentration. In other words, Sr^{++} must be pumped out of the gills against a concentration gradient. In order to test this conclusion, an experiment was run in which both the internal and external systems contained the same concentrations of 1 μg. Sr^{88} + Sr^{85}/ml. It was found that the internal concentration decreased at an O of 2.2×10^{-5} cm. sec.

The apparent active transport can be explained in one of two ways. Either strontium is in direct competition with calcium in an active pump mechanism or it is a passive response to establish electrical neutrality, due to the active uptake of Na^{+} and other ions (Krogh, 1939). Further study of this transport mechanism, as well as Sr^{++} vs. Ca^{++} relationships, are now being undertaken.

Since fish in fresh water tend to excrete water and conserve salts, and experiments have shown that fresh-water fish will accumulate strontium (Cook, unpublished data; Saurov, 1957; Schiffman, 1959) from water at a ratio of approximately 1.5:1, there must be a mechanism to convert the ionized strontium to a non-diffusible form. Protein binding in the blood is just such a mechanism. Preliminary experiments in our laboratory, using both ultra-filtration of serum and dialysis of whole blood against Ringer's solution, indicate approximately 50% or more of the blood burden is in the non-dialyzable form, probably protein-bound.

Extravascular binding sites must also be taken into account since bone appears to bind strontium as tenaciously as calcium, although the rates of binding are probably much slower than those discussed here. The high outflux rate of diffusible strontium is also reflected in the relatively inferior ability of fish to concentrate strontium in the body.

A transport system for calcium, such as this postulated for strontium, would be ideal for fish such as the steelhead trout (*Salmo gairdnerii gairdnerii*) that migrate between fresh and salt water. A rate of protein binding faster than the O for Ca^{++} would allow for an uptake of Ca^{++} limited only by the availability of calcium in the environmental water, and the number of binding sites. Once the calcium content of the environmental water is raised beyond a certain degree the binding properties of the blood and extravascular sites would become saturated and the gill would become an organ of excretion of the excess diffusible portion of blood calcium. This mode of regulation would present a picture such as found by Houston (1959). He noted a definite increase in cellular calcium but a relatively stable plasma calcium in steelhead trout when adapting to sea water. Experiments are under way to test this hypothesis.

SUMMARY

1. A perfusion technique for the study of transport across the intact gill of rainbow trout is described.

2. It was found that the outflux rate of 1.6×10^{-6} cm./sec. was greater than the influx rate of 5×10^{-8} cm./sec. for strontium, and that strontium would go out of a fish gill against a concentration gradient under these experimental conditions.

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REGENERATION OF THE ENTEROPNEUST, SACCOGLOSSUS KOWALEVSKII

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Aside from the morphological similarities which they share with the chordates (Hyman, 1959), the Enteropneusta have developed regenerative methods common to both the invertebrates and the vertebrates. The regenerative capacity of the tongue worms has been noted in early reports by Spengel (1893) in *Glossobalanus minutus,* by Willey (1899) in *Ptychodera flava,* by Cori (1902) in *Balanoglossus clavigerus* and Kuwano (1902) in *Balanoglossus misakiensis.* The first thorough investigation of regeneration was done on the Mediterranean form, *Glossobalanus* (*Ptychodera*) *minutus* by Dawydoff (1902, 1907, 1909). More recently Rao (1955) has reinvestigated regeneration in *Ptychodera flava.* A form of asexual reproduction and subsequent regeneration of tail fragments in *Balanoglossus capensis* was reported by Gilchrist (1923). All of these forms are members of a highly evolved family, the Ptychoderidae (Hyman, 1959). The current form under study is *Saccoglossus* (*Dolichoglossus*) *kowalevskii,* a species common to the shores of the North Atlantic. The only previous reference to regeneration in the present genus or its family was noted by Assheton (1908) in *Saccoglossus serpenticus,* found along the coast of Scotland.

Dawydoff (1909) discovered that when the animal was cut up into many parts, regeneration occurred from the anterior end of each fragment but never from the posterior end. Secondly, regeneration resulted either as a bud formation originating from a direct proliferation of the immediate cephalic surface (epimorphosis) or from a remodeling of the anterior portion of the amputated animal (morphallaxis). More often it was a combination of these two methods.

The factors that direct this polarized regeneration are not known. Conceivably, the anterior cut surface may inhibit regeneration from a more posterior locus. Similarly, the type of structure removed or the level of amputation may control the nature of the regenerative process utilized. These queries are approached here with an investigation of the regenerative potential of *Saccoglossus kowalevskii.*

MATERIALS AND METHODS

Living specimens of *Saccoglossus kowalevskii* were provided by the Supply Department at the Marine Biological Laboratory in Woods Hole, Massachusetts. Additional animals were collected by the author on the mud flats at Cotuit in West Falmouth, Massachusetts.

Both pre- and post-operative animals were maintained in the laboratory as follows. Individual animals were placed in small dishes (3 × 1 inches) filled with a mixture of clean sand and a small amount of organic material. The dishes were then submerged in two-inch-deep enameled pans filled with sea water. A constant flow of fresh sea water entered the pans and flowed over the submerged dishes, which were kept at a depth of one to two inches.

(The animals were difficult to keep in the laboratory except under ideal conditions. Many of the animals reported as "not recovered" in the results died from inadequate environments. They cannot survive long on glass in either standing or running sea water, nor with an excess of organic material from their natural environs.)

Since the trunk portion of the animal is rather fragile, the animals were removed for examination by first washing away the loose sand with a gentle stream of sea water. When the casting containing the animal was exposed, it was carefully lifted out and the animal dissected from its tube.

Prior to preservation, the animals were placed in a dish of clean sea water for several hours to allow removal of the digestive contents. All mucus and adherent sand were carefully stripped from the body. Anesthesia was produced by adding 85% alcohol drop-wise to the dish until the specimens were fully relaxed. Fixation was in Stockard's solution or in 10% formalin in sea water. The animals were sectioned at 10 micra and stained with Delafield's hematoxylin and Eosin-Azure II.

General Procedure. In each series, the animals were amputated transversely, with successively larger pieces being removed in succeeding experiments, beginning at the anterior end. This provided a single amputation surface on each of two animal fragments, a posterior surface on the more cranial portion and an anterior surface on the more caudal portion. The amputated animal was then returned to its dish and periodically observed for signs of anterior regeneration. The amputated cranial portions were also studied for any indication of posterior regeneration. Representative animals were fixed daily for two weeks and then at weekly intervals.

RESULTS

1. *Amputation through the proboscis*

The first regenerative challenge resulted from a transverse amputation of the proboscis at a point halfway back from the tip of the animal. Near the proximal end of the proboscis four coelomic pockets surround a median group of organs, the buccal diverticulum (stomochord), which is supported by a proboscis skeleton and encased by the glomerulus and heart vesicle. Since the amputation was anterior to these organs, only the epidermis, connective tissue and musculature of the proboscis were affected. (See Fig. 1, operation A.) Of 21 operated animals, 14 regenerated the lost portion of the proboscis within seven to nine days post-amputation. Examples of the amputated fragments and regenerates were fixed at one, two and three weeks. In some cases the junction of the new regenerate and the stump was clear because of a slight telescoping between the host animal and the regenerate. (See Fig. 2.) Several of the new regenerates exhibited various degrees of bifurcation at the tip. The degree of separation ranged from slight dichotomy to almost complete separation of the proboscis regenerate, as seen in Figure 3. In all of these cases, regeneration of the amputated proboscis occurred as a local proliferation of the tissues at the cut surface (epimorphosis) and resulted in a rapid replacement of the portions removed.

Occasionally, the amputated stump regressed instead of regenerating. Regression was noted in two cases. In one specimen examined histologically after 24 days, the original proboscis had disappeared and regression had continued to the collar. The azure-colored collar epidermis had closed the opening into the branchial chamber except for a small slit. Laterally, at the junction of the collar epidermis and the purple-staining trunk epidermis, a small proboscis formed as a bud from the trunk epidermis. (See Fig. 4.) The core of the outgrowth was filled with loose muscle fibers, connective tissues and coelomocytes.

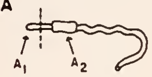
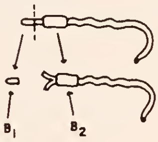
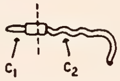
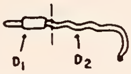
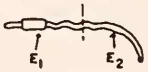
TYPE OF OPERATION	NUMBER OPERATED	ANTERIOR PIECE		POSTERIOR PIECE		REGENERATION	
		NUMBER OF:		NUMBER OF:		WITH REGRESSION	WITHOUT REGRESSION
		SURVIVORS	REGEN.	SURVIVORS	REGEN.		
A 	21	13	0	16	16	2	14
B 	12	11	0	7	5	3	2
C 	58	48	0	9	3	2	1
D 	35	29	0	4	2 ?	2 ?	0
E 	36	32	22	27	0	0	22

FIGURE 1. Comparison of regeneration originating from the anterior or posterior pieces of transected *Saccoglossus kowalevskii* after amputation at succeeding levels.

This one example indicated that in rare instances, at least, the proboscis did not regenerate directly but rather underwent a more extensive remodeling of the existing tissues that remained in the amputated animal (morphallaxis.) The exact parameters for this type of response are not known at this time.

The amputated anterior halves of the proboscises remained alive on the surface of the sand for several weeks. These isolated fragments stayed active, moved about by ciliary action, and reacted to mechanical stimulation. This response was similar to the irritability to mechanical and photic stimulation of enteropneusts reported by Bullock (1940) and Hess (1937) in similar isolated fragments. Shortly after amputation, the proboscis fragments were able to burrow into the sand, but later they lost this response. After three weeks, the pieces tended to decrease in size and developed rugate folds in the epidermis along the lateral surfaces. Multi-branched processes of solid epidermis often developed on the caudal surfaces of the proboscis fragments. However, in no instance was there any other evidence of regenerative activity in these isolated portions of the proboscis. This portion of the animal was apparently incapable of posterior regeneration or of reorganization into new individuals.

2. *Amputation through the proboscis and sagittal splitting*

In an attempt to determine the cause of the dichotomous regeneration of the proboscis, a series of 12 animals were amputated frontally through the proboscis and then the remaining stump was split sagitally to the anterior edge of the collar with iridectomy scissors. (See Fig. 1, operation B.) After five days, two animals had regressed, one to the collar region and the other to the branchial chamber. Five animals regenerated the proboscis in part. In three animals, one-half of the proboscis regenerated distally while the stump of the remaining half fused with the regenerate at the base.

Curiously, in a fourth case, the regenerated half arose from the lateral surface of the amputation stump, forming a lateral sprout from the main axis.

In two of the asymmetrical regenerates, the original organs of the proboscis had regressed. This apparently depended upon the degree of injury from the experimental incision. A new circular-shaped glomerulus, heart vesicle, and a stubby buccal diverticulum formed that extended from the proboscis stalk into the extreme proximal end of the proboscis. (See Fig. 5.) These new organs appeared to develop from the coelomocytes of the proboscis and stalk, but the exact cell precursors could not be determined from these few examples. However, each of the structures was surrounded with a very heavy non-cellular lamellated membrane, originating directly from the body of the proboscis skeleton.

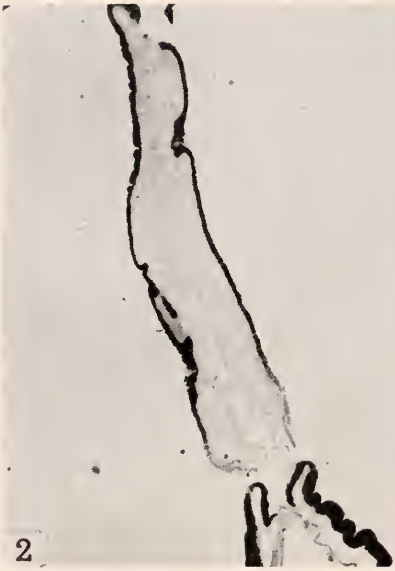
In one other example, both halves of the proboscis fused and the two regenerated together. Five other animals were not recovered.

The above results suggested that the dichotomy in the previous series was the result of regeneration from two separate loci, which sometimes fused and at other times failed to do so.

3. *Amputation through the collar*

Each animal of this group was amputated transversely through the middle of the collar, as indicated in Figure 1, operation C. The latter half of the animal, consisting of the posterior collar region and the attached trunk, was retained, as well as the proboscis and anterior half of the collar.

The amputated animal was less capable of withstanding the operation than the anterior portion that was removed. This may have been due to the trauma of operation or possibly it was a result of mucus-collection, which had a tendency to strangle the animal unless it was removed. Out of a total of 58 operated animals, only nine amputated animals were recovered. The large number of regenerative failures from the C_2 pieces was thus, in large part, due to the very high rate of mortality among the amputees. Regression of the remaining collar was quite common and usually began within two to three days after amputation. Al-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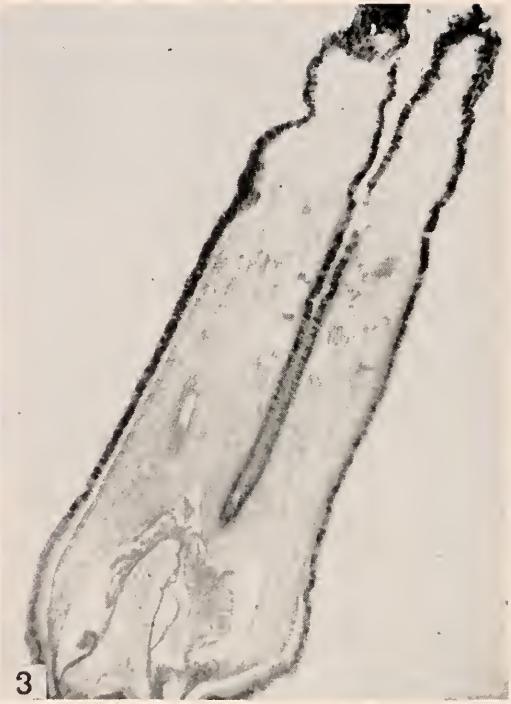
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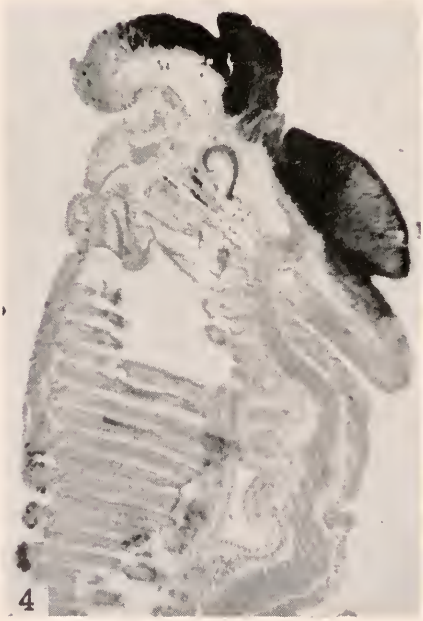
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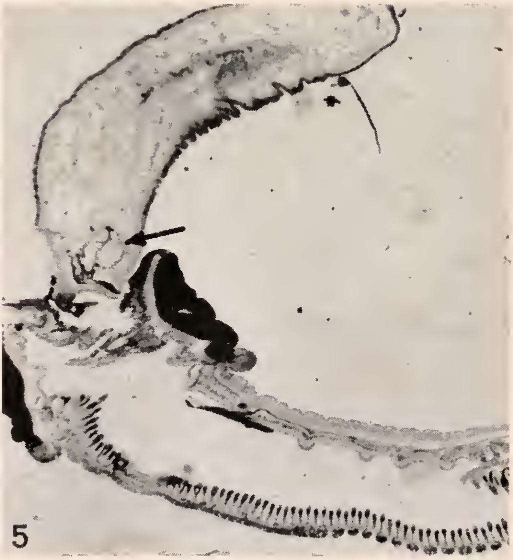
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FIGURE 2. Regeneration of the distal end of the proboscis after transverse amputation halfway back from the tip. Replacement of tissue results as a direct proliferation of the amputation surface. Fixed at seven days post-amputation.

FIGURE 3. Occasional bifurcation seen in regenerates after transverse amputation of the proboscis. The buccal diverticulum, heart and glomerulus are present in the proximal portion

most all of the operated animals regressed into the branchial region of the gonadal area within five to six days post-amputation. After regression, the animals either became stabilized or degenerated completely.

Of the animals recovered, three were found in different stages of regeneration. One had completely regenerated the amputated half of the collar and a full proboscis at 26 days post-amputation. This animal had not regressed prior to regeneration. (See Fig. 6.) Two other specimens, fixed at six days post-amputation, showed initial bud or blastema formation. The latter two cases were animals which had regressed prior to the beginning of regeneration.

The isolated pieces of proboscis and attached collar showed a gradual reduction of the attached collar between five and seven days post-amputation. Collar portions were still found 14 days after amputation but at the end of 18 days, almost all of the fragments had regressed collar parts with the denuded skeleton projecting out of the proboscis. Highly differentiated structures within the proboscis or proboscis stalk, such as heart, glomerulus, and buccal diverticulum, were retained for as long as 15 days. However, these structures disappeared from the proximal end of the proboscis, leaving only the muscle and epidermal portions of the proboscis intact. Although small buds of epidermis often appeared on the posterior surface, there was no indication of posterior regeneration. These isolated proboscises remained active and survived on the surface of the sand up to four weeks, and then gradually shrank and disintegrated.

4. *Amputation posterior to the collar*

In the fourth experiment, the animals were amputated at a position posterior to the collar so that a small portion of the branchial region was included. (See Fig. 1, operation D.) Mortality here was again very high, possibly due to both mucus constriction and the inability of the trunk region to burrow. Out of 35 operated animals, only four survived. Two of these showed no sign of regeneration and had regressed to the hepatic region at the end of nine days. The remaining two had also regressed to the posterior branchial region and were fixed at seven days. Outwardly, they had developed small, yellow fleshy bulbs at the anterior end. Each bulb consisted of a median diverticulum which formed from the mucosal lining of the old branchial region. (See Fig. 7.) However, it was very doubtful that these epidermal blebs represented anything more than local metaplasia, and they were probably not true regenerates. As shown in the next section, amputation through the identical area (the post-branchial region) often produced similar small epidermal bulbs. Histological examination of the latter growths, at three weeks post-amputation, never indicated that they differentiated further.

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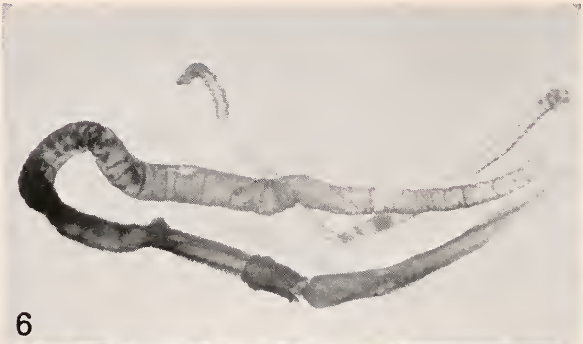
of the proboscis. Regeneration here occurs as a direct proliferation from the double amputation surfaces.

FIGURE 4. Formation of a new proboscis through morphallaxis after regression into the collar region. The proboscis is forming at the junction of the collar and trunk epidermis. Fixed at 24 days post-amputation.

FIGURE 5. An asymmetrical regenerate after amputation and sagittal splitting of the proboscis stump. A new glomerulus, heart vesicle and buccal diverticulum, indicated by the arrow, are beginning to form in the proximal end of the proboscis.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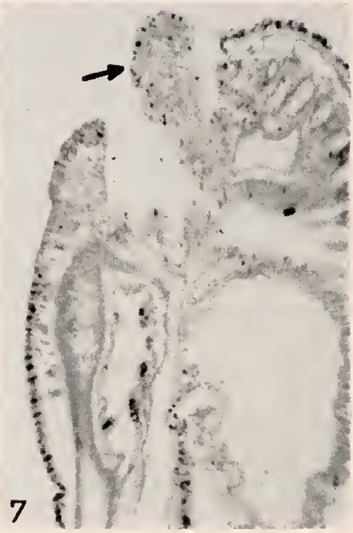
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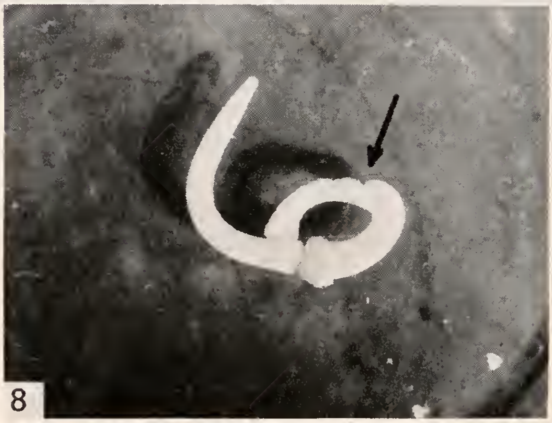
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FIGURE 6. Complete regeneration of the proboscis and anterior half of the collar after amputation through the collar. This animal had not regressed prior to regeneration but had regenerated directly from existing primordia. Fixed and cleared at 26 days post-amputation.

FIGURE 7. Sagittal view of an amputated trunk which had regressed into the posterior branchial region. The arrow indicates typical epidermal outgrowths that form on the anterior surface but never differentiate further. Fixed at seven days post-amputation.

FIGURE 8. An animal which illustrates posterior regeneration originating from an intact proboscis, collar and branchio-genital region. This young, immature animal had formed a new post branchial trunk at seven days post-amputation. Arrow indicated the level of amputation.

The amputated anterior portion, consisting of proboscis, collar and a fragment of the branchial area, was self-sustaining from five to fifteen days. The newly created caudal opening remained open and, with the original mouth opening, provided double apertures into the collar region.

No proliferation was ever found on any of the anterior pieces. The branchial chamber and collar generally persisted for a week but then began to regress thereafter. Regression usually continued until only the proboscis remained. In some cases, however, the collar and trunk were still intact at the end of three and one half weeks.

5. *Amputation posterior to the branchial region*

The last and most posterior cut was made just behind the last gill opening. The point of amputation was determined by the terminal tip of the external gill openings that taper to a point in the midline between the lateral gonads. (See Fig. 1, operation E.) Only completely intact animals were used, and both anterior and posterior pieces were kept. The anterior portion, consisting of proboscis, collar and branchio-genital region, always burrowed into the sand immediately, while the latter portion never burrowed.

At the end of three days, the anterior wound area of the posterior piece (hepatic area and caudal intestine) had healed. Of 36 operated animals, 27 pieces were still intact at the end of three days. The remainder had deteriorated. In the survivors, usually the gonadal ridges turned over to fuse with the paired, median ventral ridges, which completely sealed off the opening into the intestine. Occasionally, single or multiple transparent epidermal vesicles appeared on the healed surface, but these small blister-like growths never grew larger nor did they show further differentiation. Most of the pieces survived for seven to eight days, and at the end of one week some of these degenerated, generally starting in the hepatic region. The more anterior gonadal and posterior intestinal fragments that resulted always survived longer. In some animals, a new opening appeared in the lateral wall just behind the amputation site. Almost invariably, this was followed by an inversion of the complete trunk region behind the new opening. Thus, the entire mucosal lining was outermost while the epidermal covering now served as a lining. The posterior pieces persisted for ten days, gradually fragmenting thereafter until only long segments of the post-hepatic region remained. None of the posterior pieces had regenerated after three weeks.

For the first time in these experiments, the anterior portion, composed of proboscis, collar and branchio-genital regions, showed signs of posterior regeneration after three days post-amputation. Regeneration was first detected in the younger, immature animals but regeneration also took place in the mature animals.

As a group, the younger, immature animals had a greater growth rate than the older, mature ones. At the end of one week, each animal had developed a new post-branchial region equal in length to the intact branchio-genital region. The mean length of the regenerates at this time was 1.1 cm. (See Fig. 8.) In two animals, an entire new post-branchial trunk, equal to the animal's original length, had formed. Regional differentiation of the new trunk regenerates into gonadal, hepatic and caudal areas followed after outgrowth of the trunk. In one example, differentiation of the gonadal and hepatic regions was completed at the end of eight days.

The sexually mature animals regenerated at a slower rate than the younger forms, averaging 0.5 cm. of new growth in one week. At the end of one month, the mean length of the regenerates measured 1.6 cm. A second important difference between the immature and sexually mature animals was in the organs immediately replaced. Existing differentiated structures at the amputation surface usually accompanied the trunk outgrowths. Thus, in the short regenerates of two to three days, new gonadal tissue accompanied the regenerating trunk wall. In the females, the ovaries contained fully formed eggs, easily seen through the body wall.

Posterior regeneration was observed in 22 of 32 surviving animals. The remaining animals regressed in a manner identical to that noted in the preceding operations.

DISCUSSION

The current experiments showed that the direction of axial regeneration varied, depending upon the level of amputation. In the anterior region of the body, regeneration never originated from a posterior amputation surface, but once the level of the branchial region was passed, regeneration was possible from a posterior amputation surface. Now, however, regeneration was not demonstrable from the anterior surfaces of lower trunk areas. These results both confirmed and differed with the results obtained by Dawydoff (1902, 1907, 1909) and Rao (1955). In Dawydoff's and Rao's experiments, the animals were generally cut into about four portions, the proboscis and collar, the branchial region, the hepatic region and the post-hepatic region. They found that anterior regeneration, where technically possible, occurred from the separate fragments, but they never reported regeneration from a posterior amputation surface in *Glossobalanus minutus* and *Ptychodera flava*. One difference in the present experiments on *Saccoglossus kowalevskii* was in the method of amputation. Each operation exposed only one cut surface on each piece, oriented either cranially or caudally. It was subsequently seen that posterior regeneration can occur from an anterior piece in the post-branchial area, a fact which diverges from other reports. It is conceivable that posteriorly-directed regeneration in the latter part of the animal was prevented by a more anterior dominant regeneration surface in the earlier experiments.

Two separate phenomena seemed related to the movement of the regeneration site posteriorly. The first, a tendency toward regression of the amputated parts, was lowest in the proboscis region. It became more common in the collar region and was found quite frequently in the trunk region. Regression always involved the loss and disappearance of structures in an orderly fashion from the wound, and should be distinguished from over-all deterioration of an animal fragment. From the present data, regression did not always lead to gradual but complete disappearance of the organism. In the first three experiments, regression was occasionally arrested by unknown parameters. Subsequently, the existing tissues were reorganized and regeneration followed.

A second observation was based on the type of regeneration involved, *i.e.*, epimorphosis or morphallaxis, and its relation to the level of amputation. When the proboscis was amputated, this almost always resulted in a quick and direct replacement of the lost portion through epimorphosis, the size of the regenerate approximating the original size of the amputated part. Transsection through the collar could result in either form of regeneration, with the rapid formation of a full-size regenerate or a small regenerate undergoing morphallaxis. When the amputation was made just behind the collar, regression always followed but regeneration was not demonstrable. In the post-branchial region, posterior regeneration took place again by the process of epimorphosis.

It appears that a certain degree of controlled regression immediately precedes regeneration whenever morphallaxis takes place. Continued regression results in no regeneration and gradual deterioration of the amputated stock. The factors that initiate and control regression are still unknown.

SUMMARY

1. Transverse cuts, which separated the animal into two parts, were made on *Saccoglossus kowalevskii*. When transsection occurred through the proboscis or

collar region, the posterior animal fragment was capable of regenerating lost parts anterior to the amputation surface but the anterior animal portion was not able to replace more posterior parts.

2. When amputation was performed behind the branchial region, anterior parts were never formed from the posterior animal portion. However, regeneration of more posterior missing parts was now possible.

3. Regeneration occurred either from a direct proliferation of the tissues present at the amputation surface (epimorphosis) or through a remodeling of the anterior portion of the amputated fragment (morphallaxis).

4. As the locus of amputation was moved posteriorly, epimorphosis became less common and morphallaxis became the principal method of regeneration. Just behind the branchial region, successful posterior regeneration seemed to be accomplished exclusively through epimorphosis.

5. Immediately following amputation, regression often occured from the posterior fragments. If regeneration ensued, regression always preceded morphallaxis but did not precede epimorphosis.

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OBSERVATIONS ON THE RESPIRATORY ENZYMES OF VARIOUS LIFE-STAGES OF CHIRONOMUS PLUMOSUS, CHIRONOMUS STAEGERI, AND AEDES AEGYPTI[1]

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The pattern of respiratory enzymes in the midge genus *Chironomus* (*Tendipes*) is of interest for several reasons.[2] Most of the insect tissues investigated by Zebe and McShan (1957) exhibited very little activity of lactic dehydrogenase, an enzyme essential to the operation of anaerobic glycolysis in vertebrate tissue. Yet the larvae of some species of *Chironomus* regularly survive weeks or months in oxygen-free surroundings at the bottoms of eutrophic lakes. Furthermore, the various stages in these insects' life-histories differ markedly in their requirements for energy and in their tolerance of anoxia.

This report describes some measurements of the activity of the anaerobic glycolytic system in larvae of two species of the genus *Chironomus,* and of the succinoxidase and cytochrome oxidase systems in larvae, pupae, and adults of these species and of the mosquito, *Aedes aegypti.*

We are indebted to Dr. William H. McShan for many valuable suggestions, for the use of equipment, for assistance in choosing many of the techniques employed and for training in their use.

Materials and Methods

A. *Animal material*

The larvae of *C. plumosus* and of *C. staegeri* are found in great abundance burrowing in the bottom sediments of stratified eutrophic lakes where they are frequently subjected to several weeks or more of oxygen deprivation in late summer and fall. In the laboratory, larvae of *C. plumosus* are more resistant to anoxia than those of *C. staegeri* (Neess and Della Croce, unpublished data).

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[1] The work described here received financial assistance at various times from the United States Atomic Energy Commission, the National Science Foundation, and the Wisconsin Alumni Research Foundation, whose support is gratefully acknowledged. It is part of the material of a Ph.D. thesis submitted by the senior author to the Department of Zoology, University of Wisconsin.

[2] For a discussion of the use of the name *Chironomus* the reader is referred to Neess and Dugdale (1959).

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After a period of larval development, lasting up to two years for *C. plumosus* and for an unknown but probably shorter time for *C. staegeri,* the first indications of imminent pupation and emergence appear. At this time, the animals are marked by a darker coloration of the anterior segments and by the appearance of black pigment at the site of the adult eyes. Larvae which show no external changes preparatory to pupation will be referred to hereafter as "normal" larvae, while those in the stage just described will be designated as "pre-pre-pupational." This stage, which may last for several weeks, is followed immediately before pupation by the "pre-pupational," in which the thoracic segments become markedly swollen at the site of the future wing muscles and take on a distinctly lighter color than the rest of the animal. Often groups of pre-pupational larvae exhibit a bimodal weight distribution which Dugdale (1956) has shown to be correlated with sex, the heavier animals representing the future females.

These later larval stages are somewhat more sensitive to oxygen lack than are the earlier stages. They also seem to be more active since they are sometimes taken, apparently while swimming towards the surface, in plankton nets. In both traits, they foreshadow the pupae, which are completely intolerant of oxygen lack and which swim actively towards the surface in preparation for emergence.

The final transformation of *Chironomus* pupae into adults takes place at the surface of the lake, usually at dawn or dusk. The freshly emerged adults rest on the surface of the water briefly and then fly towards shore, where they mate during the day. The males are more active in this process, forming large swarms whose members hover rapidly for extended periods. The females rest on the ground or on leaves most of the time, but occasionally one flies through the swarm, copulates in flight, and returns to rest. At dusk the females fly back onto the lake and lay their eggs in compact sticky masses on its surface. Females which have not laid their eggs are here designated as pre-ovulatory. Those that have laid their eggs are designated as post-ovulatory. The subsequent fate of the females is not known, though the results of this investigation may shed some light on the question. The adults do not live for more than several days after emerging from the lake.

Aedes aegypti also undergoes a transformation from a less active larva through a more active pupa to a highly active adult, but it does not change in its oxygen requirements, being an obligate aerobe at all stages.

Aedes were reared from eggs kindly provided by the U. S. Public Health Service Laboratories of the Communicable Disease Center at Savannah, Georgia. Larvae and pupae of *Chironomus* were obtained by dredging bottom mud from Lake Mendota near Madison, Wisconsin, with a modified Ekman dredge (Welch, 1948, p. 176) and washing it through a wire-mesh screen with a spray of water. Adult *Chironomus* were obtained immediately after their emergence and while the females were laying eggs, by attracting them to a lantern on a boat. Swarming males and females on the ground were captured by netting. All adults were used within six hours and all larvae and pupae within two days after they were collected.

B. Methods

Enzyme activities were generally determined by Warburg manometry, using tissue homogenates. These were prepared by placing weighed live animals into a sharp-pointed ground glass homogenizer, adding ice-cold distilled water, and grinding with a motor-driven pestle. All tissues were kept in ice after being homogenized. No more than fifteen and usually less than five minutes elapsed between the time the animals were killed and the beginning of the enzyme determination. Readings of pressure changes were made at ten-minute intervals, and the average of the first four or six readings was used. Two to four animals were homogenized together to reduce the effect of individual variations in activity. Larvae and pupae were blotted on filter paper before being weighed to remove the film of water which clings to their surfaces. Adult insects were chilled to prevent them from flying before being weighed and placed in the cup.

The activity of the glycolytic enzyme system was determined by the technique of Novikoff (1948), using a 95% nitrogen, 5% carbon dioxide atmosphere. After the incubation period, the contents of one-half of the Warburg flasks were deproteinized by the addition of equal volumes of 10% perchloric acid and centrifuging, and the amounts of lactic acid formed in these flasks were then determined by the method of Barker and Summerson (1941).

Succinoxidase activities were measured by the method of Schneider and Potter (1943). In some cases, cytochrome *c* or the substrate, sodium succinate, was omitted. Cytochrome oxidase was studied principally by the method of Schneider and Potter (1943), using sodium ascorbate as the substrate. Corrections were made for the autoxidation of the ascorbate by using different amounts of tissue in the individual flasks of a series, and calculating the autoxidation by extrapolating the total oxidation to a tissue concentration of zero. A few determinations of cytochrome oxidase activity were made by the microspectrophotometric method of Cooperstein and Lazarow (1951). All chemicals used were reagent grade commercial preparations.

All the determinations of glycolytic and cytochrome oxidase activities and of the succinoxidase activities of *C. staegeri* and of *A. aegypti* were made at 38° C. Two series of measurements of the succinoxidase activity of *C. plumosus* were made. The first, which included observations on a greater number and variety of adults than the second, was made at 15° C., approximately the highest temperature which the larvae experience in their natural habitat. The second, at which all stages exhibited greater activity, was made at 38° C.

All results are expressed in terms of gas exchange or lactate production per unit wet weight of tissue. Those dry weights which were determined represented a quite constant proportion of the animals' wet weights, ranging from 13% for *C. plumosus* larvae to 26% for *C. plumosus* adults. The final concentrations of tissue in the flasks were between 0.3 and 20.0 mg. wet weight per ml. The concentrations of the different tissues used were chosen so that the changes in pressure would be of a conveniently measured magnitude.

In some cases, the concentration of tissue within the flasks was varied among replicated determinations in order to observe the effects of such variations on the activity. Succinoxidase activity proved to be related to tissue concentration in an almost linear manner in all cases in which the effect was measured. Variations in the tissue concentrations in the determinations of cytochrome oxidase were used in calculating the substrate oxidation rate, and so an assumption of linear response to such variation is necessary. In all cases, variations in unit activity caused by variations in concentration would contribute to the standard error which was calculated for the mean activity of each tissue. Therefore, it is reasonable to assume that a small standard error or a statistically significant difference between the means of two tissue types indicates that differences in concentration did not affect unit activities significantly.

RESULTS AND DISCUSSION

A. Glycolysis

The formation of lactate and the evolution of CO_2 by the larval tissue homogenates in the first series of experiments indicated the presence of a glycolytic system. The omission of both hexose diphosphate and glucose, *i.e.*, of all carbohydrate substrates, reduced the rate of lactate production by 90% while the carbon dioxide production was reduced by only 45%. This indicates that a source of hydrogen ions other than lactic acid was present in the reaction, since any reduction in pH would cause the release of CO_2 from the bicarbonate in the flasks. Since the evolution of CO_2 was prevented only by the omission of ATP from the vessels it is probable that the larvae, like the cockroach and grasshopper muscle studied, respectively, by Barron and Tahmisian (1948) and Humphrey and Siggins (1949) contain an ATP-ase capable of removing an acidic phosphate group from this nucleotide. Therefore, conclusions about the glycolytic capabilities of the larvae have been based on lactate production rather than on the evolution of CO_2.

One indication was found of a qualitative difference between the larval enzyme pattern and those found by Novikoff (1948) in tumors and Utter *et al.* (1945a, 1945b) in mammalian nervous system tissues. In the latter cases the addition of phosphorylated hexose was indispensable for the operation of the system. The rate of production of lactate of three samples of larval tissues, incubated without added HDP, was lowered by only about 40%.

B. Succinoxidase

Table I shows the means and standard errors for oxygen uptake of the various preparations. The endogenous oxidation of homogenates of samples of all the life stages of *C. plumosus* was quite low at 15°, and there were no differences among them. The effect of the omission of cytochrome *c* from the reaction mixtures was less in the less active tissues, *e.g.*, those from larvae and pre-ovulatory females (15°), while in the more active tissues the oxygen uptake was reduced by the same proportion as that found by Potter (1941) and by Schneider and Potter (1943) in rat liver under the same conditions.

At 15°, the succinoxidase activity of homogenates of larvae of *C. plumosus* was little greater than their endogenous oxidation. The pupae showed a highly significant increase in activity over the larvae and a highly significant difference between the sexes, the male pupae having an oxygen uptake over twice that of the female. The rate of oxygen uptake of freshly emerged adult males was over twice that shown by the male pupae, and that of actively swarming males was slightly higher yet. However, the activities of freshly emerged female adults showed no similar increases, but rather fell to levels approaching those of larvae.

Harvey and Beck (1953) and McShan, Kramer and Schlegel (1954) had also found differences in succinoxidase activity between the sexes in an insect, the cockroach *Periplaneta americana*, and Barron and Tahmisian (1948) found a sexual difference in the over-all oxygen uptake of *Periplaneta* thoracic muscle. In these instances the ratio of oxidative activity in the male to that in the female was only two or three, whereas adult male chironomids displayed a rate ten times that of the females. At first it was thought that this difference was correlated with the fact that the difference between the activities of the sexes is greater in *Chironomus* than in *Periplaneta*. This explanation was shown to be incorrect when homogenates of females which had laid their eggs were found to have a succinoxidase activity some seven times higher than homogenates containing unlaid eggs.

TABLE I

Succinoxidase activity in cm.^{3}/gm. wet weight/hr.

Life stage	*C. plumosus*, 15°	*C. plumosus*, 38°	*C. staegeri*, 38°	*A. aegypti*, 38°
Larvae				
"Normal"	.43 ±.05 .13* .41 ±.15**	1.59 ±.06 .45**	2.92 ±.10 2.66 ±.12 (sw.)	.72 ±.01 (2 days after hatching) 1.51 ±.22 (5 days after hatching) 2.35 ±.07 (6–13 days after hatching) .68 ±.15 (6–13 days after hatching)* .91 ±.51 (6–13 days after hatching)**
Pre-pre-pupational, ♂		1.84 ±.08		
Pre-pre-pupational, ♀		1.89 ±.08		
Pre-pupational, ♂		2.24 ±.10 0*	3.60 ±.04	
Pre-pupational, ♀		1.94 ±.10 0*	3.32. ±11	
Pupae, ♂	2.54 ±.18 .12* 1.09**	12.26 ±.28	9.22 ±1.15	2.79 ±.09 (♂ + ♀) .91 ±.04* (♂ + ♀) .65 ±.04** (♂ + ♀)
Pupae, ♀	1.13 ±.04	8.24 ±.34	7.21 ±.28	
Adult, ♂				
Freshly emerged	5.85 ±.32 .15 ±.02* 2.35 ±.33** 11.7 ±.23†			15.29 ±.61
Swarming	7.65 ±.93 4.20 ±.37**			
With eggs	.42 ±.03			
Adult, ♀				14.82 ±.17
Freshly emerged	.59 ±.23 .18** 6.91 ±.80†			12.04 ±.41 (after blood meal)
Pre-ovulatory	.46 ±.05 .45 ±.06**	2.08 ±.66 16.70 ±3.3†		
Post-ovulatory				
Lake caught	1.43 ±.40			
Land caught	3.79 ±.26 2.43 ±.46**			

* =no substrate; ** =no cytochrome; † =heads and thoraces; (sw.) =swimming.

The hypothesis that the eggs of *C. plumosus* were capable of inhibiting the activity of the succinoxidase enzyme system was supported by the fact that homogenates of thoraces, alone, of pre-ovulatory females displayed a succinoxidase activity of the same order of magnitude as homogenates of the entire post-ovulatory animals, which suggested that there was no change in the enzyme system itself at the time the eggs were laid. The inhibitory effect of the eggs was directly demonstrated by homogenizing an egg mass, collected immediately after it had been laid, together with adult male midges in a ratio of two parts by weight of egg mass to five parts of whole male insect. This was approximately the ratio of the weight of a gravid abdomen to the weight of an adult female. The homogenate of male adult with eggs had an activity as low as that of the entire pre-ovulatory females, while a control without eggs showed a typically high activity. Since the egg masses made up only 40% of the weight of tissue present, while their presence reduced the succinoxidase activity by about 90%, it seems probable that they were actively inhibiting the enzyme system and not merely diluting it with inert tissue.

As Table II indicates, the developed egg masses, when homogenized with rat liver in the same proportion as with male *Chironomus,* reduced its activity by 70%, both at 15° and at 38°, but the egg-containing abdomens of the females, similarly homogenized, had no such effect. This difference may well be related to changes in the chemistry of the egg masses following fertilization or oviposition. The difference between the response to the undeveloped eggs by the tissues of the females and by the rat liver, and the greater magnitude of the effect of the eggs on the adult males may indicate a specific difference in the properties of the enzymes of the two forms. It remains to be seen whether the inhibiting effect of the egg masses has any functional significance for the insects.

TABLE II

Inhibition of mammalian succinoxidase; activity expressed as cm.^{3} O_2*/gm. wet weight/hr.*

Tissue and temperature	Control	With *C. plumosus* egg-laden abdomen	With *C. plumosus* egg masses
Rat liver 15°	4.06	4.02 ± .27	1.09 ± .52
Rat liver 38°	25.68 ± 1.34	21.04 ± 1.26	4.89 ± .65
		With *A. aegypti* egg-laden abdomen	With *A. aegypti* eggs
Mouse liver 38°	32.49 ± 1.39	41.05 ± .05	30.34 ± .38

Female midges captured on the shore were readily identifiable as pre-ovulatory or post-ovulatory by the shape of their abdomens, those of the former being swollen and round in cross-section while those of the latter were flat and narrow. The succinoxidase activity of all the members of the former group was distinctively low and that of the latter high. The condition of the midges captured on the lake was more ambiguous. A number of these animals were classified as post-ovulatory whose abdomens were not as completely reduced as those of the animals on shore or which were taken immediately after they were seen to lay eggs. The succinoxidase activity of this group of "post-ovulatory" females was significantly lower than that of the animals taken on land and it showed greater variability. These results seem to indicate that the female midge lays a number of egg masses in separate places on the lake and then returns to shore.

The elimination of the inhibition by the egg masses did not entirely destroy the difference between the sexes, but it did reduce it to the same order as that found in other insects. The rate of oxygen uptake of homogenates of entire swarming males was twice that of the post-ovulatory females. The same ratio was found when the activities of only the heads and thoraces were compared. The absolute values of the latter were 50–80% higher than those of the entire animals. These results might be expected in view of the very high metabolic rates generally displayed by the thoracic flight muscles of insects. Another indication that the muscles of insects may vary in the level of enzymes of the Krebs cycle was reported by Brooks (1957), who found differences between the succinoxidase activities of different muscles of individuals of the American cockroach as well as between those of the sexes.

At 38°, the activity of the larval succinoxidase was four times that at 15°, indicating a Q_{10} for the system of about two. There was no endogenous oxidation at the higher temperature. Pre-pre-pupational larvae at 38° showed a significantly higher activity than "normal" larvae. There was no difference between the mean activities of the heaviest and lightest individuals of this group, though, as suggested above, they very probably represented female and male. The mean activities of pre-pupational larvae were higher still, and a small, though not significant, difference between the sexes was found. As at 15°, the male pupae showed a sevenfold increase in activity over the larvae, and the female pupae a somewhat smaller increase. The activities of homogenates of whole pre-ovulatory adult females were again little more than those of the larvae, while those of homogenates of thoraces alone were eight times as high.

Tissues from pre-pupational larvae of *C. staegeri*, like those of *C. plumosus*, were somewhat more active than those from "normal" ones, and those from the males were more active than those from the females. The pupae were more active still, but the difference between the activities of pupae and of pre-pupational larvae was less than in *C. plumosus*. The difference in activity between male and female pupae was not significant.

The rate of oxygen uptake by the preparations of *staegeri* larvae was almost twice that shown by preparations of *plumosus* larvae, but the activity of *staegeri* pupae was less than that of *plumosus* pupae. One group of *staegeri* larvae which were taken in a plankton net showed a lower than average rate of succinoxidase activity, perhaps indicating that swimming behavior begins earlier in this form than in *plumosus*.

Since the *Aedes* used in this work were reared in the laboratory from the egg, the development of their enzyme activities could be followed from an earlier stage than that of *Chironomus*. Larvae examined two days after hatching showed a low level of activity, scarcely higher than that of the endogenous oxidation. After five more days of development at a temperature of ca. 20°, the activity had doubled and after eight days, had levelled off at a rate some three times higher than the original one. This final rate was between those shown by larvae of *C. plumosus* and *C. staegeri*.

The omission of cytochrome *c* from homogenates of either larvae or pupae reduced the activity to a level barely above that of the endogenous respiration. The latter was four times higher at a temperature of 38° than the endogenous respiration of *C. plumosus* larvae at 15°.

The pupae of *A. aegypti*, which were not differentiated by sex, had a succinoxidase activity only slightly higher than that of the larvae. The activity of the adults was some six times as high as that of the larvae or pupae and showed no sexual difference.

After a blood meal, the activity per unit weight of the females declined slightly but this may be due to the lower activity of the ingested blood which made up a substantial part of the weight of the insect. Two attempts were made to measure any possible inhibition of succinoxidase by *Aedes* eggs. In the first, mouse liver was homogenized together with dry and presumably viable eggs. In the second, mouse liver was homogenized together with the abdomens of female mosquitoes which had received a blood meal. In neither case was the succinoxidase activity of the mouse liver treated with eggs or abdomens reduced below that of untreated homogenates.

C. *Cytochrome oxidase*

Table III shows the cytochrome oxidase activity of the various homogenates in cm.^{3} oxygen taken up per gram wet weight per hour. These activities are in all cases greater than the succinoxidase activities of the same tissues, the ratio between the two varying between three and ten. The changes in activity of cytochrome oxidase during development follow much the same pattern as do those in succinoxidase activity, but there is no evidence for an inhibition of cytochrome oxidase by the developing eggs of *C. plumosus*. The greater individual variations made some of the results less clear-cut.

TABLE III

Cytochrome oxidase activity at 38° in cm.^{3}/gm. wet weight/hr.

Life stage	*C. plumosus*	*C. staegeri*	*A. aegypti*
Larvae			
"Normal"			14.97 ± 1.68
10 meters and deeper	7.70 ± .38 1.99 ± .35** 0*	21.48 ± .61	
9 meters and shallower	13.03 ± .42		
Pre-pupational, ♂	15.89 ± 2.62		
Pre-pupational, ♀	7.13 ± 2.43		
Pupae, ♂	37.90 ± .60	57.07 ± 9.28	25.86 ± .06 (♂ + ♀)
Pupae, ♀	35.75 ± .75	42.16 ± 6.56	
Adult, thorax, ♂	119.70 ± 18.50		

* = no substrate; ** = no cytochrome.

The "normal" larvae of *C. plumosus* fell into two distinct groups. The majority showed an oxygen uptake of 7.7 ± 0.4 cm.^{3}/g./hr. One group, taken in late September from a relatively shallow area of Lake Mendota in which higher temperatures and oxygen concentrations are found during summer than in the habitat of the first group, had an uptake of 13.0 ± 0.4 cm.^{3}. These larvae showed no external sign of imminent pupation, though animals from this area of the lake generally pupate earlier than those deprived of oxygen earlier in the season. This may indicate either that previous oxygen deprivation reduces the activity of larval cytochrome oxidase or else that changes in cellular respiration preparatory to pupation may precede visible morphological changes.

Male pre-pupational larvae showed a slightly higher cytochrome oxidase activity than the "normal" group from shallow water; females showed a lower, though not significantly lower, activity. As with succinoxidase, the cytochrome oxidase activity of the pupae was some five times higher than that of the larvae and the activity of the male adults was three times that of the male pupae. There was no significant difference between the activities of male and female pupae.

C. staegeri and *A. aegypti* larvae displayed, respectively, three and two times the cytochrome oxidase activity of *C. plumosus* larvae, but the *staegeri* pupae showed only 1.3 times the activity of *plumosus* pupae, and the activity of *Aedes* pupae was less than that of *plumosus* pupae. It is of some interest that both the increases in activity of the two oxidative enzyme systems studied and the reduction of tolerance to anoxia which accompany pupation are greatest in *C. plumosus,* intermediate in *C. staegeri,* and least in *A. aegypti.*

Additional Observations

A small number of observations on the oxidation of reduced cytochrome *c* by homogenates of *C. plumosus* and *C. staegeri* larvae were made using the microspectrophotometric method of Cooperstein and Lazarow (1951). These measurements were made at room temperature, *i.e.*, ca. 25° C. *C. staegeri* was found to have the more active cytochrome oxidase by this method as well, but the difference between the two species was not so great as when measured manometrically.

The effect of cyanide on the total oxidative metabolism of some of the subjects was studied in two ways. The oxygen uptake of intact larvae of *C. staegeri* and *A. aegypti,* maintained without food in a synthetic lake water medium, was measured by Warburg manometry at 20° C. and the effect of concentrations of sodium cyanide varying from 10^{-2} to 10^{-5} M was noted. The effect of 10^{-4} and 10^{-5} M cyanide on the oxidation of reduced cytochrome *c* by homogenates of larvae of *Chironomus* was also observed.

The addition of 10^{-4} M NaCN to their medium reduced the oxygen uptake of live larvae of both species by 30–50%, and higher concentrations caused proportionally greater reductions. Cyanide at 10^{-4} M concentration completely blocked the oxidation of cytochrome, and 10^{-5} M cyanide reduced it by 70%.

The ability of *C. plumosus* larvae to maintain a normal level of oxygen uptake in the presence of low external concentrations of oxygen, and the extremely low oxygen tensions at which the hemoglobin dissolved in their hemolymph unloads oxygen (at .00079 atmospheres, or 0.6 mm. Hg, according to Fox, 1945) has led to the suggestion (Prosser, 1952, p. 323) that the oxidative enzyme pattern of the larvae may be modified to permit the transfer of oxygen at very low concentrations.

To test this suggestion, two Warburg measurements of cytochrome oxidase were conducted under an atmosphere of 1% oxygen and 99% nitrogen. Under these conditions, no oxygen was taken up, indicating that if an enzyme modification such as that suggested exists, it probably involves a circumvention of cytochrome oxidase rather than an increase in its efficiency or affinity for oxygen. This possibility is also suggested by the fact that some insects are resistant to strong concentrations of cyanide. For example, the strongly aerobic larvae of caddis flies have been observed in this laboratory to survive in 10^{-2} M NaCN for more than twenty-four hours. Furthermore, Schneiderman and Williams (1954) have directly demonstrated a cytochrome oxidase by-pass in the diapausing silkworm pupa. Since much of the respiration of the *Chironomus* larvae studied here was eliminated by treatment with cyanide, it seems that the terminal oxidases of insects display considerable variety.

SUMMARY

1. Larvae of *Chironomus plumosus* and *Chironomus staegeri* have an active glycolytic enzyme system.

2. The succinoxidase and cytochrome oxidase systems of *C. plumosus, C. staegeri*, and *Aedes aegypti* are least active in the larval stage, more active in the pupal stage and most active in the adult stage. The increase in activity from larval to pupal stage is greater in those species, *C. plumosus* and *C. staegeri*, in which the larva, but not the pupa, is resistant to anoxia than in *A. aegypti*, in which neither is resistant.

3. Male pupae and adults of *C. plumosus* have a higher rate of succinoxidase activity than females. No sexual difference was found between the cytochrome oxidase activities of pupae or between the succinoxidase activities of *A. aegypti* adults.

4. The developing eggs of *C. plumosus* exert an inhibitory effect on the succinoxidase system of the insect and of rat liver.

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PHOTOPERIODISM AND THE ANNUAL TESTICULAR CYCLE OF THE BOBOLINK (DOLICHONYX ORYZIVORUS), A TRANSEQUATORIAL MIGRANT, AS COMPARED WITH TWO TEMPERATE ZONE MIGRANTS[1]

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It is now quite generally accepted that seasonal changes in day-length play an important role in the temporal regulation of several annual cyclic phenomena in many birds of the northern hemisphere. Best known is the effect of photoperiods on the annual cycle of the testes, but evidence is accumulating that there is an effect also on a metabolic cycle apparently peculiar to migratory species, an effect perhaps on migration and perhaps also on the molt cycle (summarized in Farner, 1959).

Transequatorial migrants are of special interest in generalizing from known photoperiodic effects because they are exposed annually to two day-length cycles rather than one (*cf.* Fig. 1), and yet, like all other birds which breed above tropical or subtropical latitudes, they have only one reproductive period each year. Unfortunately, very little experimental work has so far been done on transequatorial migrants. Marshall and Serventy (1959) showed that photoperiods apparently do not influence either the gonadal or molt cycles of a shearwater which, following a breeding season off southeastern Australia, migrates across the equator to the North Pacific Ocean, as far as the Aleutian Islands. Preliminary studies (Engels, 1959) indicated a photoperiodic effect on the testicular cycle of the bobolink (*Dolichonyx oryzivorus*), which breeds in North America above Lat. 40° N. and after a transequatorial migration to South America occupies an area between approximately Lat. 10° and 30° S. These preliminary experiments were in part deficient in design, and certain conclusions drawn from them must be modified in view of the further experimental results now to be reported.

MATERIALS AND METHODS

Sixteen bobolinks (*Dolichonyx oryzivorus*), 6 slate-colored juncos (*Junco hyemalis*), and 6 white-throated sparrows (*Zonotrichia albicollis*) were used in the experiments. The juncos and white-throated sparrows, representative of Temperate Zone migrants, were captured in February and March from populations wintering in the vicinity of Chapel Hill, North Carolina, and were held through the following summer in an outdoor aviary. Seven of the bobolinks were caught in May on their northward migration, one near Raleigh, N. C., the others near Chapel Hill. Like the juncos and white-throated sparrows, these were held in an outdoor aviary through the summer and thus were exposed to nearly normal

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day-lengths until the beginning of experimental lighting on October 1. Nine of the bobolinks were captured in early September near Wilmington, N. C., while on their southward migration, just a few weeks before the start of experimental lighting. Five of the 7 birds taken in May were returned to the aviary in the following May, after 7 to 8 months of artificial day-lengths during which they passed through a complete testicular cycle. They spent the summer again in the outdoor aviary and were used in further experiments beginning in the next autumn—in the tables and discussion these birds are referred to as "second-year experimentals." (Since 5 of the 16 bobolinks were each used twice, there are 21 entries in Table II.)

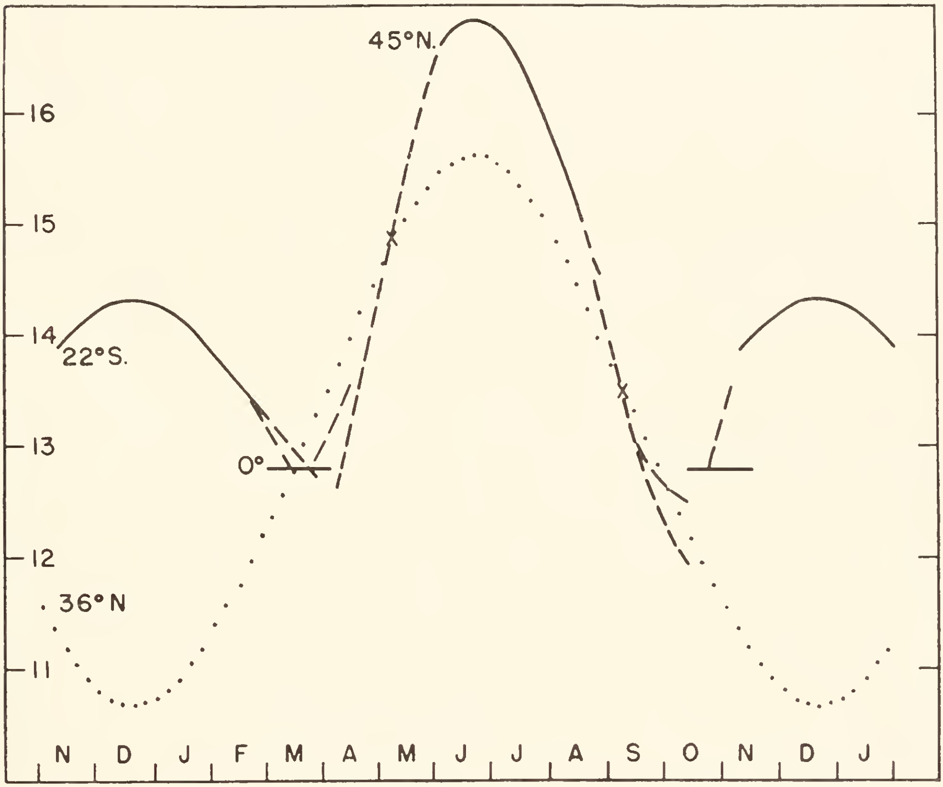

FIGURE 1. Annual cycles of day-lengths (hours between beginning of morning civil twilight and end of evening civil twilight) experienced by bobolinks: *solid lines,* near the center of their "wintering" grounds, at Lat. 22° S.; near the center of their breeding grounds, at Lat. 45° N.; and at the equator, Lat. 0°, during their transequatorial passages; *broken lines,* during migrations (approximate); *x*, time of capture of spring and fall migrants (early May and early September) in North Carolina; *dotted line,* the cycle (at Lat. 36° N.) experienced by captive bobolinks in aviary at Chapel Hill, N. C.—the general form of this cycle is that experienced by all Temperate Zone migrants as well as by non-migrant residents of middle latitudes. Curves for hours between sunrise and sunset would be lower by about one hour, varying from about ¾ hour at the equator and at Lat. 22° S. in December to about 1¼ hours at Lat. 45° N. in June. (From data in: Tables of Sunrise, Sunset and Twilight: Supplement to the American Ephemeris, 1946; U. S. Naval Observatory. Government Printing Office, Washington, D. C.)

TABLE I

Responses of juncos and white-throated sparrows to 14-hour photoperiods following 8 weeks (October 1–November 26) of shorter periods; photoperiods from March to October 1 were those natural at Lat. 36° N.

Species	Photoperiod Oct. 1–Nov. 26	Date killed	Vol. of testes	Spermatogenesis
Junco	10-hour	Jan. 16	295 mm.³	sperm in bundles
Junco	10-hour	Jan. 16	247	sperm in bundles
Junco	10-hour	Jan. 16	230	sperm in bundles
Junco	12-hour	Jan. 16	1.2	inactive
Junco	12-hour	Feb. 6	2.1	inactive
Junco	12-hour	March 6	1.2	inactive
White-throated sparrow	10-hour	Jan. 16	440	sperm in bundles
White-throated sparrow	10-hour	Jan. 16	403	sperm in bundles
White-throated sparrow	10-hour	Jan. 16	226	sperm in bundles
White-throated sparrow	12-hour	Jan. 16	2.4	inactive
White-throated sparrow	12-hour	Feb. 6	8.2	spermatocytes
White-throated sparrow	12-hour	March 6	3.1	inactive

Different groups of birds, two to four in each group, were exposed to experimental photoperiods beginning October 1, as follows: 10 hours daily (10 hours light, 14 hours dark) continuously for 7 to 8 months; 14 hours daily, also continuously; 10 hours, 11 hours, or 12 hours daily for eight weeks, followed by 14 hours daily until May (about 6 months); and, in an outdoor aviary, changing day-lengths normal to Lat. 36° N.—this latter regime may properly be termed an experimental treatment for transequatorial migrants since it differs so radically from that which they would have encountered in the free state (*cf.* Fig. 1).

The conditions of confinement during the experimental treatment for the juncos, white-throated sparrows and those bobolinks caught during May were similar to those used in the preliminary experiments (Engels, 1959); that is, two to four birds together in a cage measuring 24 × 24 × 36 inches. The other bobolinks, including the second-year experimentals, were confined individually in Hendryx "finch breeding cages" measuring 8 × 9 × 16 inches. Terramycin was added to the drinking water supplied to these birds. Food, during most of the experiments, consisted of a chick laying mash (Purina Layena); during the last few months we changed to a mash prepared for "game" birds (also Purina).

The development of black pigment in the beak was taken as the criterion of testicular recrudescence in the bobolinks. The first appearance of this pigmentation probably coincides with that stage of the spermatogenic cycle when most of the spermatocytes are undergoing the first meiotic division. However, it should be noted that the observations have to do with evidence of the cycle of production of male sex hormone, which apparently directly causes beak pigmentation (Engels, 1959), and only by inference with the spermatogenic cycle.

EXPERIMENTAL RESULTS

The results of the experiments as given in Tables I and II may be summarized as follows:

Juncos and white-throated sparrows

Valid comparison may be made between these Temperate Zone migrants (Table I) and the bobolinks caught in May and subjected to similar artificial photoperiods (Table II, spring captures, Groups B and D). Exposed to 14-hour photoperiods after 8 weeks of 10-hour photoperiods, juncos and white-throated sparrows had already completed spermatogenesis by January 16, whereas bobolinks first developed beak pigmentation between mid-February and early March, 4 to 7 weeks later. On the same 14-hour photoperiod following 8 weeks of 12-hour photoperiods, testes of juncos and most white-throated sparrows remained in the winter inactive state at least to early March, by which time bobolinks had already developed beak pigmentation.

TABLE II

Response of male bobolinks to various photoperiods; beak pigmentation indicates production of male sex hormone and, inferentially, progress of spermatogenesis at least to first (meiotic) division of spermatocytes. ("Spring captures" and "2nd-year experimentals" experienced the natural day-lengths of Lat. 36° N. for at least four months before start of experiments on October 1)

Group	Category	Photoperiods		Beginning of beak pigment*
		Oct. 1–Nov. 26	Dec.–May	
A	autumn cap.	10-hour	10-hour	May 16
	2nd-yr. exp.	10-hour	10-hour	May 11
B	spring cap.	10-hour	14-hour	Feb. 13
	spring cap.	10-hour	14-hour	Feb. 20
	autumn cap.	10-hour	14-hour	March 12
	autumn cap.	10-hour	14-hour	March 16
	2nd-yr. exp.	10-hour	14-hour	Feb. 17
C	spring cap.	11-hour	14-hour	Feb. 27
	spring cap.	11-hour	14-hour	Feb. 27
D	spring cap.	12-hour	14-hour	Feb. 20
	spring cap.	12-hour	14-hour	March 6
	autumn cap.	12-hour	14-hour	March 2
	autumn cap.	12-hour	14-hour	March 16
	2nd-yr. exp.	12-hour	14-hour	Feb. 17
E	autumn cap.	14-hour	14-hour	—**
	autumn cap.	14-hour	14-hour	—**
	2nd-yr. exp.	14-hour	14-hour	Feb. 17
F	autumn cap.	Natural to Lat. 36° N.		April 20
	autumn cap.	Natural to Lat. 36° N.		April 20
	spring cap.	Natural to Lat. 36° N.		April 1
	2nd-yr. exp.	Natural to Lat. 36° N.		April 1

* Dates indicate end of interval (4 to 7 days) during which beak pigmentation appeared.
** Beak still neutral, May 25.

Bobolinks

Lumping together the three categories in Table II (spring captures, autumn captures, second-year experimentals) for the various groups, we may note the following significant points. When exposed to constant 10-hour photoperiods (Group A), beak pigmentation did not appear until mid-May. When, after 8 weeks on 10-hour photoperiods, the length of the photoperiod was increased to 14 hours, beak pigmentation developed in the various individuals between mid-February and mid-March (Group B), two to three months earlier than in the first group. Similarly, when the 14-hour photoperiod was preceded by 8 weeks of either 11-hour or 12-hour photoperiods, beak pigmentation appeared variously from mid-February to mid-March (Groups C, D). However, on continuous exposure to 14-hour photoperiods from October 1 (Group E), without prolonged previous exposure to short days, only one of three birds (a second-year experimental) developed pigment in the beak; two birds caught a few weeks before treatment began still lacked beak pigmentation in late May, when the experiment was terminated. Bobolinks held in the outdoor aviary (Group F) developed beak pigmentation in April, definitely later than most of the birds on artificial photoperiods, but from 4 to 7 weeks earlier than those continuously on 10-hour photoperiods. Song was heard from the aviary beginning in the latter part of March, when several inches of snow lay on the ground.

In general, beak pigmentation began somewhat later in birds caught in the autumn than in the other categories.

DISCUSSION

Some preliminary experiments on bobolinks (Engels, 1959) seemed to show that the rate of response to 14-hour photoperiods depended inversely on the length of the photoperiod to which males had been exposed previously during the postnuptial, photorefractory phase of their cycle. That is, on 14-hour photoperiods beak pigmentation developed earlier in males which had been previously exposed to 10-hour photoperiods, several weeks later in males previously on 12-hour photoperiods. The difference is not apparent in the present experiments. However, birds used in the earlier study had been held on continuously long photoperiods (16-hour) throughout the summer, rather than on natural day-lengths as in the present study, and it is suggested that this difference in preliminary treatment is responsible for the difference in results. The idea is held, tentatively, that the reinforcement of photorefractoriness induced by some months of previously long days was overcome sooner on the shorter (10-hour) photoperiod. Further study is necessary to determine if we have to do here with any facet of the photoperiodic mechanism as operative in nature.

The relatively rapid response of juncos and white-throated sparrows to long photoperiods in early winter following a few weeks of short photoperiods in autumn (Table I) is characteristic of Temperate Zone migrants. Bobolinks treated in identical manner, however, were quite slow to show a response (Table II, B–D). It would appear that the cycle in these transequatorial migrants strongly resists the accelerating influence of long days, and that some process in the mechanism of response even on long days requires a rather long time, several weeks longer than in Temperate Zone migrants. Farner (1954) expected such a retardation of testicular recrudescence in response to long days on the part of transequatorial migrants, and he postulated for them (p. 29) "a characteristically long refractory period." Although we may get involved in semantic difficulties here, the present experiments suggest rather that the retardation could be due, at least to some considerable extent, to a very slow rate of response following the termination of refractoriness. In other words, the value of Farner and Wilson's (1957) constant *k* for testicular growth may be very much lower in bobolinks, especially on long photoperiods, than in Temperature Zone migrants.

Wolfson and Westerhoff (1960) in a short summary report on photoperiodic responses of *Dolichonyx* conclude, as had I previously (Engels, 1959), that a preparatory phase (= photorefractory phase) exists in this transequatorial migrant and that, moreover, this phase "may be regulated by the short days during the period of the autumnal equinox." They further conclude that "compared with temperate species, the duration of the period of short days required may be longer." This seems to me to be an unnecessary extrapolation and quite probably an inaccurate estimate of the operation of the mechanism in nature. In all Temperate Zone migrants so far investigated, the refractory period is terminated in nature by mid-November (Farner, 1954, p. 30; Farner, 1959, p. 732). Study of Figure 1 will show that it would be virtually impossible for migrating bobolinks to continue to experience beyond mid-November the short days of low or middle latitudes north of the equator. (See also final paragraph of this discussion.)

It may be of interest to note especially the responses of those bobolinks designated in Table II as second-year experimentals. These are the same birds which a year earlier were the "spring captures" in Groups B, C and D: as such, following several months of experimental lighting, they all had gone through a relatively sudden post-nuptial molt in May, with accompanying disappearance of the black pigmentation characteristic of the beak in nuptial dress. In nature these events would not have occurred until about two months later. After another summer, then, in the outdoor aviary, they were again exposed to artificial photoperiods as "second-year experimentals." The three which had been distributed one each in Groups B, D and E developed beak pigmentation almost simultaneously during the 7-day interval ending February 17. I would not emphasize the relatively early date nor the coincidence of response (both of which may have some significance), but rather the appearance of beak pigment (also full song) in the one second-year experimental of Group E. This was the only one of 7 male bobolinks (including four birds of the preliminary experiments—Groups A, G; pp. 761, 762, Engels, 1959) on continuously long photoperiods which developed beak pigmentation. We may at least suspect that the abnormally long interval since the last testicular regression, perhaps together with the relatively shorter days of late summer and early fall (see also last paragraph, below), permitted this bird to pass through the refractory phase and eventually to respond to the stimulus of the 14-hour photoperiod.

Despite the obvious difficulty of interpretation of these second-year experimentals, it seems to me that the general picture revealed in these experiments is one which fits the requirements of a photoperiodic mechanism as the timer for annual recrudescence of the testes in a transequatorial migrant: (1) the initiation of those processes leading to release from photorefractoriness, during the relatively short days of early northern autumn and during the southward passage through the equatorial region; (2) following eventual release from photorefractoriness, regrowth of the testes at a slow rate during the southern hemisphere summer when day-lengths, though declining, are still relatively long; correlated (3) with processes, the regulation of which is largely unknown, leading to migration. Migration itself exposes the birds, once past the equator, to photoperiods of rapidly increasing length (*cf.* Fig. 1).

We may note here that bobolinks held captive in the northern hemisphere (Lat. 36° N.) and exposed to natural day-lengths (and temperatures) showed a revival of the testicular cycle by April (Table II, F). The first flocks of bobolinks (all males) normally arrive from the south at this latitude about May 1, at about the time at which our locally wintering juncos and white-throated sparrows begin their northward migration. Thus, the testicular cycles of these captive bobolinks were approximately in phase, and it may be assumed that if the birds had been released, they could have reached the breeding ground at about the proper time and in breeding condition. It is therefore suggested that, as far as the photoperiodic mechanism is concerned, bobolinks are not required to "winter" below the equator; they could establish a wintering population in the northern hemisphere. The reverse would not seem to be true of such Temperate Zone migrants as juncos and white-throated sparrows, without readjustment of their photoperiodic mechanisms. Both by reason of their inability to overcome refractoriness on intermediate day-lengths (about 12 hours; *cf.* Table I) and their rapid response to long days when released from refractoriness, they probably could not extend their wintering range to and beyond the equator and still maintain that regularity of the annual cycle which leads to the coincidence of one annual reproductive period with a favorable climatic season.

Finally, it should be pointed out that bobolinks may usually not experience such a long period of such relatively short days as used in the present experiments, especially if any appreciable part of the morning and evening twilight periods adds to the effective length of the natural photoperiod (Fig. 1), as is almost certainly true. If so, they must be able to pass through the photorefractory phase and become photoreceptive on photoperiods even longer than 12 hours. It will be of interest to test this possibility.

SUMMARY

1. Bobolinks exposed to 10-hour photoperiods daily through the fall, winter and spring did not develop the beak pigmentation characteristic of the male nuptial dress until early in May. Others exposed at the same time to 14-hour photoperiods daily failed to develop beak pigmentation. But when exposure to 14-hour photoperiods was preceded by 8 weeks (October–November) of 10-, 11- or 12-hour photoperiods daily, pigmentation of the beak began variously between mid-February and mid-March.

2. Juncos and white-throated sparrows exposed to 14-hour daily photoperiods following 8 weeks (October–November) of 10-hour daily photoperiods had completed spermatogenesis by mid-January; but in others for which the preliminary photoperiod had been 12 hours the testes remained inactive.

3. Bobolinks held throughout the fall, winter and spring in an outdoor aviary, on changing day-lengths natural at Lat. 36° N., developed beak pigmentation in April, thus being approximately in phase, in their testicular cycles, with locally wintering juncos and white-throated sparrows.

4. The results are interpreted to mean that bobolinks, unlike typical Temperate Zone migrants, are able to overcome post-nuptial photorefractoriness on days of intermediate length, such as would be encountered during transequatorial migration, but that regrowth of the testes through the long days of the southern hemisphere summer proceeds at a rate much slower than that demonstrated by Temperate Zone migrants on similarly long days, and that full recrudescence is thus delayed, probably beyond the onset of northward migration.

5. It is further suggested that as far as the photoperiodic mechanism is concerned bobolinks could establish a wintering population in the northern hemisphere; but juncos and white-throated sparrows could not extend their wintering range into the southern hemisphere without readjustment of this mechanism.

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LUNAR ORIENTATION OF ORCHESTOIDEA CORNICULATA STOUT (AMPHIPODA)

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The celestial navigation accomplished by Talitridae (Amphipoda) was first reported by Pardi and Papi (1953) and Papi and Pardi (1953). These two papers contain numerous observations of the solar navigation of *Talitrus saltator* and, in the second paper, certain night-time observations suggested the existence of lunar navigation in this species, *i.e.,* appropriately time-shifted angles of orientation with the moon. The existence of solar navigation has been confirmed with several other species of Talitridae: *Talorchestia megalophthalma* and *Talorchestia longicornis* (Menaker, 1958); *Talorchestia deshayesei* (Pardi and Grassi, 1955); *Orchestoidea corniculata* and *Orchestoidea benedicti* (unpublished qualitative observations of the author). The existence of lunar navigation, however, had not been completely documented until the recent paper by Papi and Pardi (1959) which presents results supporting the contention that, under the experimental conditions described, *Talitrus saltator* oriented to moonlight with an angle that varied with lunar position. This resulted in a relatively constant and ecologically "correct" compass orientation. On the basis of this evidence, the authors conclude (p. 596) "dass zwei verschiedene physiologische Rhythmen die Sonnen- und die Mondorientierung von *Talitrus* bedingen." (Two different physiological rhythms are responsible for the solar and lunar orientation of *Talitrus.*)

Because of the significance of such a conclusion to the general theory of endogenous rhythms, the present study was undertaken to determine whether *Orchestoidea corniculata* Stout, a related amphipod which is capable of solar navigation, is similarly able to navigate by moonlight.

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METHODS AND MATERIALS

The experimental animals were adult and sub-adult specimens of *Orchestoidea corniculata* Stout, collected usually on the morning before the experiment. In a few cases, as indicated in Table I, the animals were collected one or two weeks before the experiment and kept at constant temperature until the day of the experiment. Following collection, all animals were kept in light-tight, one-liter bottles containing moist sand. The animals can survive two weeks in such containers with no evidence of oxygen shortage. No control of the temperature was attempted on the day of the experiment, but the range was not greater than 16° to 28° C. Collections were made on the beach in front of the Scripps Institution of Oceanography, La Jolla, California. The shoreline there is oriented north 15° east; the "correct" azimuth orientation to return the animals to the water is thus 285° from north.

In order to avoid fog, city sky-glow, surf sounds, etc., the experiments were conducted in a level, open field about 40 km. northeast (inland) of San Diego.

Table I

Orientation of Orchestoidea corniculata with moon: animals kept in constant darkness

Date	Time	Lunar stage: days	Lunar azimuth	Orientation azimuth	Vector length	N	Angle with moon
1960							
*9 July	2241	16	137°	258°	0.74	26	121°
9 July	2306	16	141°	266°	0.77	32	125°
**9 July	2330	16	148°	277°	0.40	28	129°
***10 July	0055	16	171°	260°	0.62	29	89°
*10 July	0133	16	183°	280°	0.64	30	97°
10 July	0153	16	189°	307°	0.62	25	118°
*10 July	0330	16	216°	001°	0.64	26	145°
10 July	0355	16	222°	349°	0.65	29	127°
16 July	0144	22	093°	212°	0.59	30	119°
30 July	2003	8	228°	352°	0.53	31	124°
30 July	2055	8	239°	348°	0.56	25	109°
30 July	2105	8	241°	359°	0.62	23	118°
30 July	2147	8	248°	010°	0.58	26	122°
30 July	2158	8	250°	041°	0.65	13	151°
6 August	2201	15	144°	314°	0.72	24	170°
7 August	0005	15	181°	299°	0.85	25	118°
7 August	0017	15	185°	293°	0.61	26	108°
7 August	0240	15	224°	329°	0.65	25	105°
7 August	0252	15	226°	338°	0.79	29	112°

* Animals kept for one week at 20° C.
** Animals kept for one week at 10° C.
*** Animals kept for two weeks at 10° C.

The observation chamber was an opaque, white, circular plastic tray 25 cm. in diameter, 4 cm. deep, over which an unmarked sheet of glass was placed. No attempt was made to control the humidity within the chamber; Papi and Pardi (1959, p. 585) state that this is usually unimportant.[1]

The camera was mounted 1.4 meters above the chamber. Photographic lighting involved a single electronic photo-flash bulb ("strobe light") fixed 1.2 meters above the observation chamber, and at a constant position relative to it. The results suggest that the photographic equipment did not affect orientation. As later discussed, the flashes necessary for picture-taking did have an effect.

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[1] See, however, Papi and Pardi (1953, p. 501, 502).

A compass and a clock, out of view of the animals, were included in each photograph.

Results are summarized by the method described by Pardi and Papi (1953, p. 463): a vector sum of the positions of the individual animals is calculated (using 16 equal sectors), and this is divided by the number of animals. The orientation of the resultant vector gives the average orientation direction and its length gives an indication of the degree of scatter about the average. Such a vector can range in length from zero, indicating an even distribution of directions around the center, to unity, indicating all animals within the same sector. Random distributions will, of course, result in vectors larger than zero.

In order to determine the vector length which would result from random, independent orientation of the animals, 20 "experiments" were performed by assigning positions to 30 hypothetical "animals" by use of a table of random numbers. Eighty per cent of the resultant vectors were less than 0.30 in length and none was greater than 0.37. This suggests that a vector of length 0.35 or less based on the positions of 20 to 35 animals, the number usually used in the actual experiments, is of doubtful meaning. As there is clearly a lack of independence in the behavior of the individual animals, which predominantly cluster around the edge of the container, and often crowd and crawl over one another, a vector of greater length, perhaps 0.50 or more, is required to indicate definite orientation. No observations were used in the tests of the lunar navigation hypothesis (Tables I and II) if the calculated vector was less than 0.40 in length. The interaction invalidates conventional statistical techniques for more rigorously evaluating variability on the basis of an individual experimental result.

TABLE II

Orientation of Orchestoidea corniculata *with moon: animals not kept in constant darkness*

Date	Time	Lunar azimuth	Orientation azimuth	Vector length	N	Angle with moon
1960						
6 August	2015	122°	288°	0.59	25	166°
6 August	2025	124°	291°	0.87	21	167°
6 August	2225	151°	339°	0.77	25	188°
6 August	2235	153°	299°	0.72	25	146°
*6 August	2245	156°	319°	0.87	25	163°
*6 August	2255	159°	325°	0.86	30	166°
7 August	0025	187°	321°	0.68	22	134°
7 August	0045	193°	286°	0.47	31	93°
*7 August	0100	198°	325°	0.77	25	127°
*7 August	0110	201°	321°	0.75	26	120°
7 August	0305	229°	280°	0.44	27	51°
7 August	0315	231°	298°	0.64	28	67°
*7 August	0325	233°	341°	0.64	26	108°
*7 August	0335	235°	320°	0.52	25	85°

* "Redarkened" animals; all others "Natural Light."

All angles are measured clockwise; azimuths are measured clockwise from north.

RESULTS

Moonlight as the orienting stimulus

In order to determine whether the observed orientations were due to moonlight, a group of amphipods, which had shown a given orientation relative to the moon, was shaded and an image of the moon was presented at about 180° from the actual position of the moon by means of a mirror. The results, shown in Figure 1, clearly demonstrate that the apparent lunar position is the dominant orienting stimulus for *Orchestoidea corniculata* under these experimental conditions.

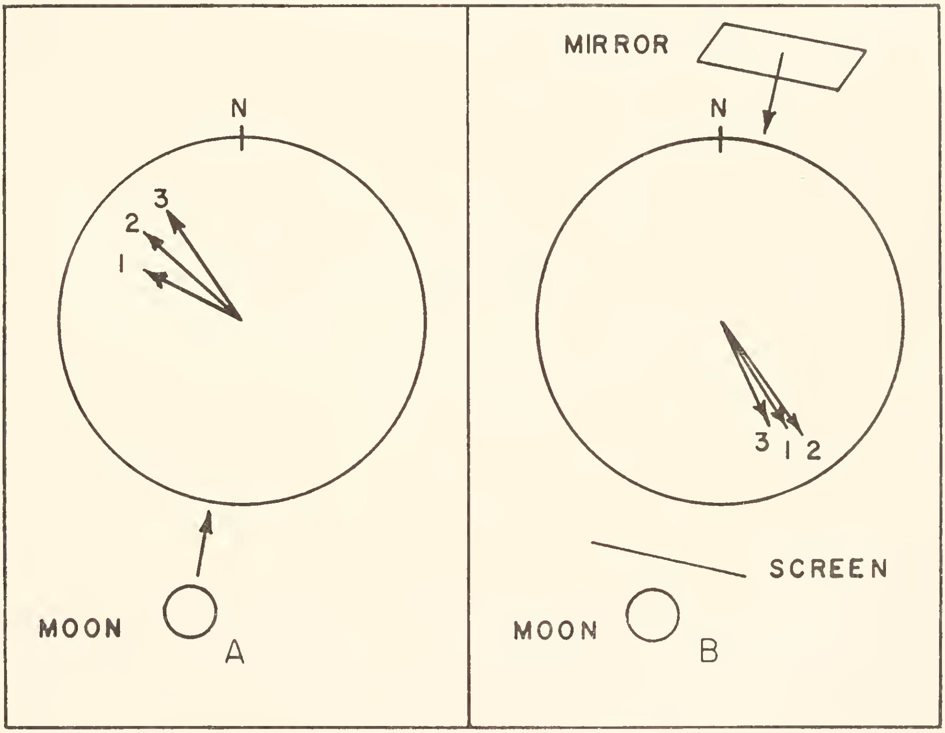

FIGURE 1. Orientation of a group of *Orchestoidea corniculata* to apparent lunar position. A: Initial orientation with the moon; B: orientation with moon reflected at about 180° from true position. Arrows within circle (which has unit radius) indicate average direction and scatter of orientation. Numbers adjacent to arrows indicate sequence in which pictures were taken. Observations made 10 July 1960, 0153–0209 hours, with 25 amphipods.

Serial observations with electronic flash

In initial experiments, a series of three photographs of each group of animals was taken. It was noted that groups of amphipods frequently showed a consistent shift in average direction of orientation, presumably as a reaction to the electronic flash. These results, while too few to be statistically significant, indicated a trend for the animals to orient with successively larger angles with the moon. (See, for example, Fig. 1A.) There was significant evidence of an increase in scatter from Photograph 1 to Photograph 3. For example, in 19 sets of 3 photographs, Photograph 1 showed least scatter in 14 cases and Photograph 3 showed most scatter in 12 cases.

Two series of ten photographs were taken to determine whether the shifts in direction and increase in scatter are cumulative (Fig. 2). In both cases, the ultimate angle of orientation with the moon after ten photographs was much smaller than the initial angle (contrary to the initial trend, over sets of only three photographs), with a net shift of 85° to 90°. In neither case is the final orientation more "correct" than the initial (although an average of the vectors of Figure 2B would be much closer to "correct" than either initial or final results). The short duration of the experiments, less than 15 minutes in each case, precludes the possibility that the shift is due to some normal compensation for changes in lunar position. Furthermore, the shift apparently does not take place without photography: qualitative continuous observation of a group of unphotographed amphipods, kept in the observation chamber for 15 minutes, indicated no noticeable orientation shift. (2 September 1960, 2315–2330 hours; lunar day 12; lunar azimuth 206°–210°; estimated orientation azimuth, initial and final, about 340°; angle with the moon, about 130°.)

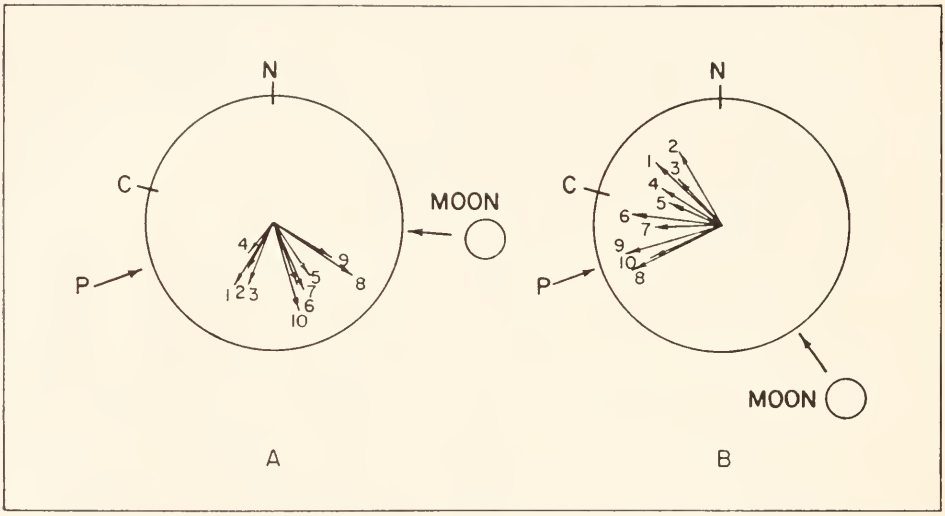

FIGURE 2. Orientation in sequential photographs of two groups of amphipods. A: 16 July 1960, 0144–0158 hours, with 30 amphipods; B: 6 August 1960, 2201–2214 hours, with 24 amphipods. Arrows within circle (unit radius) represent average direction and scatter of orientation; numbers associated with arrows indicate sequence. "P" indicates direction of photoflash; "C" indicates "correct" orientation direction relative to "N," north.

The results in Figure 2 seem best interpreted as a resetting effect of light on a rhythmic system, a phenomenon previously demonstrated using similar millisecond electronic flashes[2] (Pittendrigh, in press). The lengths of vectors in

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[2] See Pardi and Papi (1953, pp. 466–467) for an alternative explanation of a superficially similar result with solar orientation.

Figure 2 indicate that, after an initial increase in scatter over the first three to five photographs, there is a recovery so that final orientation, after ten photoflashes, is strongly directed, but in a different direction from the initial.

Because of these complications with multiple photographic observations of the moonlight orientation of *Orchestoidea corniculata*, all data subsequently cited consist only of results from first photographs of the initial, unshifted orientation, and no group of amphipods was used twice.

Orientation of amphipods kept in constant darkness

Observations of the orientation of *Orchestoidea corniculata*, kept in complete darkness until just prior to observation, are summarized in Table 1 and Figure 3. As Figure 3 shows, the data can be explained by the hypothesis that this amphipod, when exposed after ten or more hours of constant darkness, orients to the moon

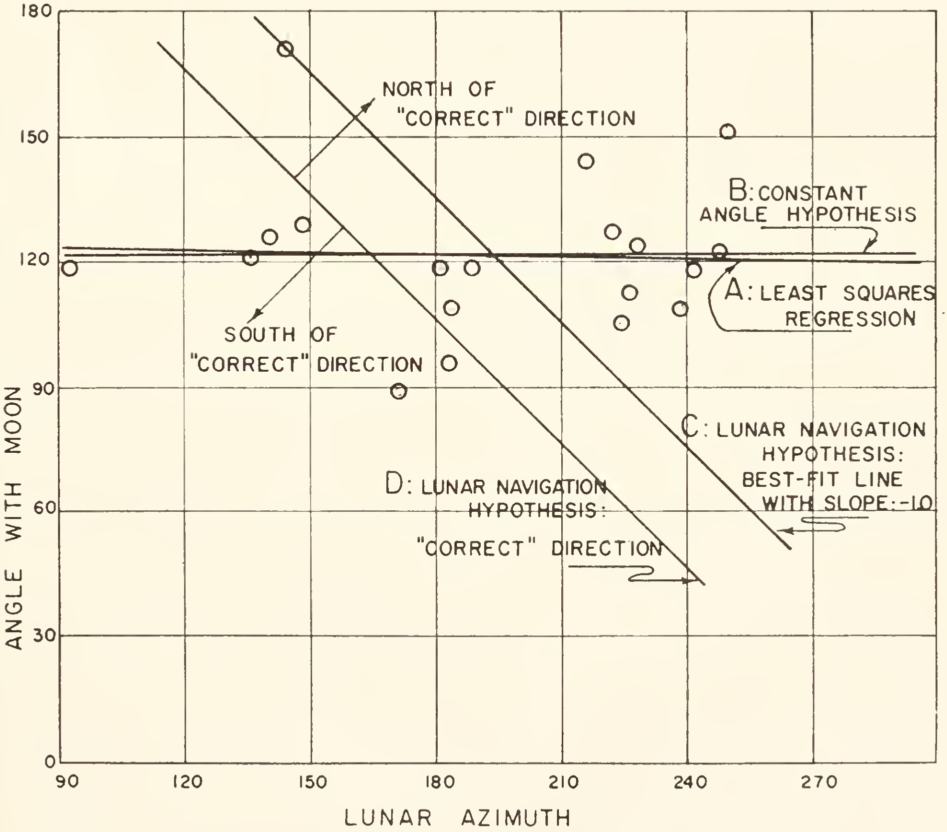

FIGURE 3. Orientation of *Orchestoidea corniculata* with moon, compared with three hypotheses: animals kept in constant darkness. The slope of the least-squares regression line (A) is -0.015. This is not significantly different from zero (line B); ($p > .50$). The probability that the slope of the regression line is as large as -0.50 is less than .001; steeper slopes (-1.0 for line C) are even less likely; and the departure from line D is even greater.

with an average angle of about 120°, regardless of lunar stage or position (Lines A and B). The hypothesis of a *continuously-operating* endogenous lunar physiological rhythm in this species, similar to that claimed for *Talitrus saltator,* appears unnecessary and is contradicted by the data (Lines C and D).

Orientation of amphipods exposed to sunset and moonrise

Additional observations were made with amphipods placed in constant darkness at the time of collection, and then re-exposed, in the sand-containing bottles, to

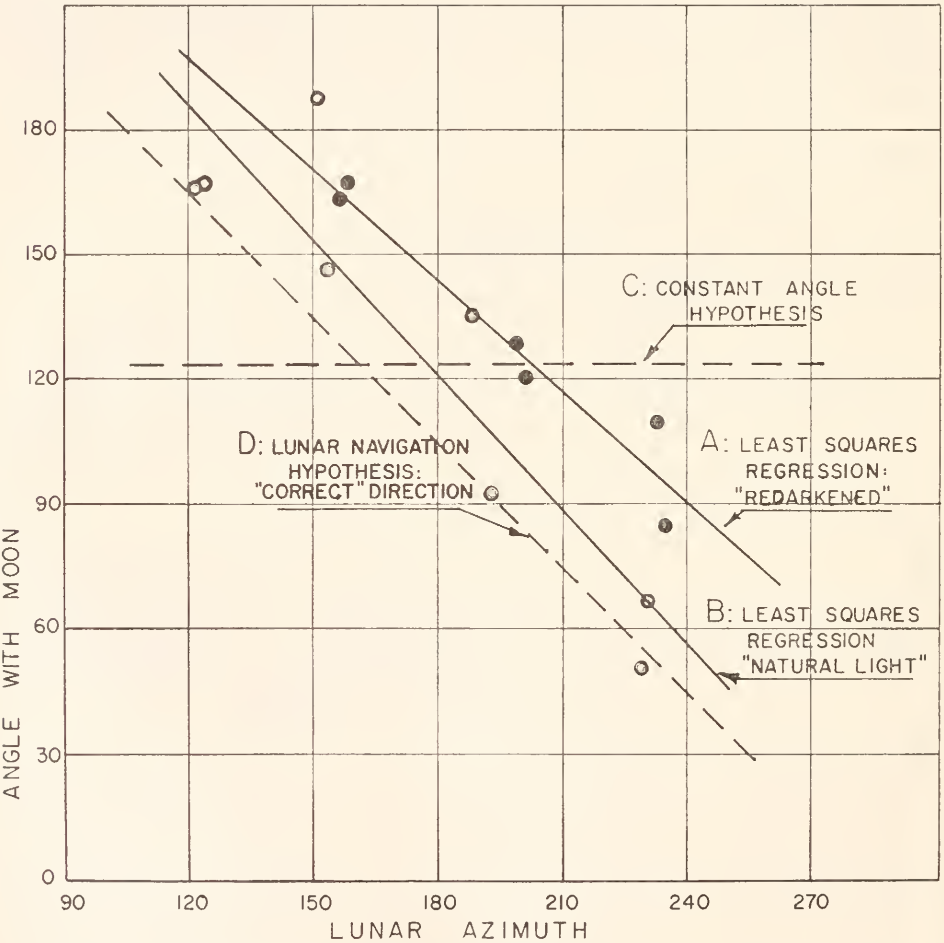

FIGURE 4. Orientation of *Orchestoidea corniculata* with moon as a function of lunar azimuth: animals not kept in constant darkness. Open circles represent orientation of "Natural Light" animals; solid circles represent orientation of "Redarkened" animals. The slope of line A is −0.888; and of line B is −1.078. The probability of the slope being −1.00 is > .20 for line A, and > .50 for line B. Points to the right of line D are north of "correct" orientation.

natural light about one hour prior to sunset. Those which were then continually exposed until observation are designated "Natural Light"; others which were replaced in constant darkness two hours after moonrise are designated "Redarkened." The observations, made August 6 and 7 (full moon), are summarized in Table II and Figure 4. The orientation resulting from this treatment of the animals does not seem adequately explained by the constant-angle hypothesis (Line C); the probability that the slope of the regression is 0 is extremely small in both cases ($p < .001$).

The directions of orientation, particularly those of the "Redarkened" animals, are generally somewhat north of the "correct" 285° azimuth, but the slopes of both regression lines are not significantly different from − 1.0, the expected value if the animals compensated appropriately for shifts in lunar position.

A possible explanation is that sunset and/or moonrise (or perhaps removal from constant dark) can initiate a single cycle of appropriately time-compensated lunar orientation in these organisms. Such a system, with no information carry-over from the preceding nights, would afford the animals a crude but workable orientation mechanism (except at first quarter?). It would not require the organizational complexity and long-term precision necessary for the *continuously-operating* endogenous lunar periodicity claimed for *Talitrus saltator.* Further investigation may indicate that "night-sun" orientation (Pardi, 1953/54) or some other by-product of solar navigation is involved. No investigation of the effect of this treatment on the solar navigation of the amphipods was made.

SUMMARY

1. The orientation of *Orchestoidea corniculata* in direct moonlight, compared with the orientation when moonlight is reflected from a mirror, demonstrates that apparent lunar position is the dominant night-time orienting stimulus for this organism.

2. An analysis of the effects of repeated photographic recording of the orientation indicates that this species changes its angle of lunar orientation and the scatter about the average as a result of repeated electronic photo-bulb flashes.

3. Using only single observations of each group of animals, in order to avoid these artifacts, it was determined that this species, kept in total darkness for ten or more hours before observation, orients at a relatively constant angle with the moon, regardless of lunar stage or position. This result is not compatible with the hypothesis that *O. corniculata* possesses a *continuously-operating* lunar physiological rhythm similar to that claimed for *Talitrus saltator.*

4. When the amphipods were exposed to sunset and moonrise on the night of observation, there was an indication of subsequently time-shifted angles of orientation with the moon. The tentative hypothesis of a single-cycle night-time orientation rhythm, re-initiated by the appropriate stimuli each night, would explain such observations.

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SEX DIFFERENCES AND ENVIRONMENTAL INFLUENCE ON DOPA-OXIDIZING ACTIVITY IN DROSOPHILA MELANOGASTER[1]

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Horowitz and Fling (1955) have described a rapid method for determination of tyrosinase activity in Drosophila. The method involves grinding adults, centrifuging, and decanting the supernatant as enzyme source. Colorimetric readings of the dopa-oxidizing activity are made to obtain enzyme-substrate reaction curves. The authors found that the maximal rate of dopachrome formation at 30° C. is directly proportional to enzyme concentration. This rapid method was used in a series of studies by the writer because it made possible quick repetition of determinations and thereby allowed for on-the-spot analysis of dopa-oxidizing activity during metamorphosis. (The exact nature of the enzymes involved is not known. Danneel (1943) has described three phenolases in Drosophila: one, a monophenolase which converts tyrosine to dopa, another a diphenolase which converts dopa to dopachrome, and a third which converts dopachrome to melanin.)

In attempting to apply this method, the writer obtained very different activity levels among 50-mg. samples of larvae and adults, even when these were taken from the same batch. It was found that modification of the method (as described below) eliminated some of the variation in activity rates between different samples. However, much variation still remained, and it was suspected that sex of the adults and larvae, and exposure of larvae to relatively dry air of the air conditioned laboratory were influencing the subsequent rate of dopa-oxidizing activity. The following study was undertaken to determine whether these factors do influence the rate of activity.

MATERIALS AND METHODS

The flies used were those collected from a wild population at Beaufort, N. C. (see Jacobs, 1960). The strain is a light tan form, similar to Canton-S, made "isogenic" for chromosomes 1, 2, and 3 by means of a dominant inversion j90 marker stock from Dr. H. J. Muller and a C1B stock from Dr. M. Whittinghill.

The culture medium used was a modification of the Carpenter-Baker formula containing: 1000 cc. water, 40 g. white ground cornmeal, 15 g. agar, 40 g. dried Fleischmann's yeast type 2019, 1 g. KH_2PO_4, 8 g. Rochelle salt, 100 cc. white Karo syrup, and 0.5 g. of: NaCl, $CaCl_2$, $MnCl_2$ and $FeSO_4$. The medium was seeded with an aqueous suspension of dry active Fleischmann's yeast.

About 50 cc. of medium were placed in each half-pint milk bottle. To such

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[1] This study was aided by the Lalor Foundation.

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bottles were added 30 female and 10 male parents. Each day, the parents were transferred to a fresh bottle to which ten fresh females and two males were added.

All cultures were kept at 25° C. in the dark. When the late larvae crawled up the sides of the bottles, as they do before pupation, the largest were removed and sexed, a few at a time, under water, on the basis of gonad size (see Bodenstein, 1950). Emergence tests of some of these larvae showed that the reliability of the sexing technique was excellent. For each enzyme determination, approximately 200 of the sexed larvae were placed in each of two 55 × 55 mm. specimen jars. One of these jars was placed, without a lid, in a 250-mm. Scheibler desiccator in which a saturated solution of $K_2CO_3 \cdot 2H_2O$ was kept in order to produce 43% R. H. (relative humidity) conditions (see Lange, 1939). The other jar was fitted with a lid with a hole 45 mm. in diameter cut in it and a disc of wire gauze fit in it, to prevent larvae from crawling out of the jar, which is possible when the walls are wet. This second jar was placed in a desiccator containing distilled water. The two desiccators were placed in the dark in a G. E. refrigerator kept at 24.5° C. with a Cenco 46042 heater thermostat unit. After the desiccators had stood for four hours, 50 mg. of the largest larvae (to the nearest whole larva) were used for each enzyme determination.

Adults were obtained by etherizing them within an hour after emergence from the pupa cases, while they were still translucent and just after expansion of the wings. Before the adults were used for the colorimetric enzyme determinations, the heads were removed so the eye pigments would not interfere with the tests. It took about five minutes to remove the heads from the 50 mg. of flies used in each determination. The flies were weighed before removal of the heads.

The method for dopa-oxidizing activity was as follows: from the 100% R. H. desiccator were weighed 50 mg. of larvae (to the nearest whole larva). These were washed with distilled water into a powder funnel, to the bottom of which was fitted a wire gauze. The funnel was then inverted over the open end of a 13 × 100 mm. Corning #7725 tissue grinder tube sawed off to a length of 45 mm. so it would fit the high speed head of the centrifuge. Distilled water was streamed onto the wire gauze to wash the larvae into the grinder tube. This water was pipetted out and replaced three times, after which all free water was pipetted from the inside of the tube, and the outside was wiped dry. The tube with the larvae was then weighed. The larvae were ground in the tube in 1 ml. of phosphate buffer (pH 6). To the tube was immediately added 0.5 ml. of L-dopa (0.02 *M* aqueous), and the tube was placed in a water bath at 30° C. for ten minutes. The tube was then placed into ice water for one minute while 1 ml. of chloroform was added. The tube was then removed, Parafilm was held over the open end, and the tube was shaken a few seconds so the lipids would dissolve in the chloroform, for lipids were found to cause turbidity which interfered with colorimeter readings. The tube was then placed into the high speed head of the centrifuge (0° C.) and brought to 6,000 *g* in one minute, left there for five minutes, then reduced to stopping in one minute. Into a ½-inch tube of the B & L Spectronic 20 colorimeter were placed 0.5 ml. of the supernatant and 2 ml. of the phosphate buffer. A reading was taken against a distilled water standard at 475 millimicra, and the tube was placed in the water bath at 30° C. for ten minutes, after which another reading was made.

An equal number of larvae were removed from the 43% R. H. desiccator and treated as were the 100% R. H. larvae except that after the tube with the larvae was weighed, water was added to bring the total weight up to that shown by the tube with the 43% larvae. (In the study of unsexed larvae kept under the two conditions as described in the next paragraph, in which the larvae were never in water, the 43% R. H. larvae were found to weigh an average of 0.833% less than the 100% R. H. larvae.) Adults were added unwashed to the grinder tube, ground in buffer, and treated the same way as were the larvae.

When it was found that larvae at 43% R. H. showed increased dopa-oxidizing activity as compared with those at 100%, the question arose as to whether the 43% larvae would also pupate at a faster rate. To test this, 200 unsexed larvae were placed in the jar in each desiccator and allowed to remain for four hours as before, after which 20 of the largest larvae in each desiccator were weighed, boiling water was poured into the jars, and the pupae were counted. This was repeated ten times for the four-hour period and ten times for an eight-hour period.

RESULTS

The results of the tests showed larvae kept at 43% R. H. had greater dopa-oxidizing activity than those kept at 100% (Table I). For the larvae at 43%, the colorimeter readings taken just after centrifuging, as well as those taken ten minutes later, showed lower per cent of transmittance than did those for the larvae kept at 100% R. H. Since this drop in per cent of transmittance is sufficient to show the relative activity rate, this figure alone will be considered in this discussion for the sake of simplicity, and will be referred to as activity in colorimeter units.

TABLE I

Dopa-oxidizing activity of Drosophila melanogaster *as indicated by colorimeter readings of reactant mixtures of tissue homogenates and dopa substrate. A low per cent of transmittance indicates a high rate of dopa-oxidizing activity*

Type of flies	Number of 50-mg. samples	Per cent of transmittance		
		Reading no. 1 (after centrifuging) Mean ± S.E.	Reading no. 2 (10 min. after no. 1) Mean ± S.E.	Difference between no. 1-no. 2 readings Mean ± S.E.
Larval females in humid conditions (100% R. H.)	15	70.86 ± 2.42	59.66 ± 3.47	11.20 ± 1.63
Larval females in dry conditions (43% R. H.)	15	60.00 ± 2.06	42.40 ± 3.04	17.60 ± 0.92
Larval males in humid conditions (100% R. H.)	13	76.07 ± 2.77	66.61 ± 2.45	9.46 ± 1.83
Larval males in dry conditions (43% R. H.)	13	66.38 ± 3.58	51.61 ± 3.97	14.77 ± 1.58
Adult females	53	59.77 ± 0.97	51.67 ± 1.24	8.10 ± 0.52
Adult males	38	85.60 ± 0.23	84.00 ± 0.34	1.60 ± 0.71

Larval females at 100% R. H. showed 11.20 units, while those at 43% R. H. showed 17.60 units of activity. Larval males at 100% R. H. showed 9.46 units, while those at 43% showed 14.77 units. Adult females showed 8.10 units, while adult males showed only 1.60 units. It is thus observed that the larvae at 43% R. H. showed greater activity than those at 100%, and adult females showed greater activity than adult males. All these differences are significant well below the 5% level. It appears that larval females show greater activity than larval males, but the observed "t" value for this difference is small (1.52) and above the 5% level of significance.

For both larvae and adults, the average wet weight of females was greater than that of males (Table II). It took an average of only 25.52 larval females to weigh 50 mg., while it took 30.53 males. Likewise, it took only 39.87 adult females, while it took 55.14 males.

In the counts of larvae kept at 100% and 43% R. H. for four hours, there were 381 pupae in the 100% jars and 352 in the 43% jars. The chi-square value for this difference is only 1.147, which is not significant. More significant results were obtained in the eight-hour samples, in which 674 pupae were counted in the 100% jars and only 594 in the 43% jars. The chi-square value for this difference is 50.473, which is significant below the 1% level. Thus, contrary to what was expected, the larvae at 100% R. H. pupated at a faster rate than those at 43% R. H.

TABLE II

Mean number of flies in 50-mg. samples taken to the nearest whole fly. The same number of larvae were used in the 100% and 43% relative humidity samples

Type of flies	Number of 50-mg. samples	Number of flies per 50-mg. sample (Mean ± S.E.)
Larval females	15	25.52 ± 0.06
Larval males	13	30.53 ± 0.05
Adult females	53	39.87 ± 0.07
Adult males	38	55.14 ± 0.09

DISCUSSION

The observation that *D. melanogaster* females show more dopa-oxidizing activity than males raises the question as to whether this activity is involved in general metabolism, perhaps as a result of egg production. This would seem reasonable in view of the fact that differences in gonad size, as observed among male and female larvae, are less than the difference in size between the reproductive structure (egg masses) of the adult female as compared with the small mass of the adult male testes: and difference in dopa-oxidizing activity among male and female larvae is also smaller than the difference in activity between male and female adults. Bodine and Allen (1938) have demonstrated, in grasshopper eggs, a tyrosinase proenzyme which can be activated by shaking and other means. Kucera (1934) has found *D. melanogaster* adult females to consume more oxygen than adult males. However, Sussman (1952) found no difference in oxygen uptake in *Platysamia cecropia* moth diapausing larvae injected with tyrosinase inhibitors, as compared with controls, and he suggested that tyrosinase is not a terminal oxidase in those larvae.

The increased rate of dopa-oxidizing activity in the larvae at 43% R. H. might be due to control by Weismann's ring gland, which in turn is regulated by the nervous system. Dennell (1949) has inhibited tyrosinase activity in *Calliphora erythrocephala* by destruction of the gland. In this fly, in spite of the presence in the blood of both enzyme and substrate, no oxidation of tyrosine takes place until the pupation hormone controlled by the gland is liberated. Dennell discovered a glucose dehydrogenase system which declined in activity as the larvae approached pupation, while tyrosinase activity increased. Dennell suggests that the dehydrogenase system is controlled by the gland and inhibits tyrosinase activity.

The classical view of the role of phenolase activity in insects is that certain phenolic oxidation products of tyrosine are responsible for tanning and hardening of the larval cuticle to form the puparium. (See Dennell, 1958a, for review.) It was this theory which prompted the present study in which pupae were counted following killing with boiling water. It was thought that the increased phenolase activity would cause increased rate of puparium formation. But, contrary to what was suspected, the larvae at the lower humidity (43%), in which dopa-oxidizing activity was accelerated, showed a slower pupation rate than those at 100%. This decreased rate at lower humidity has not yet been explained.

The failure to find an increase in pupation rate to accompany an increase in dopa-oxidizing activity in larvae at the lower humidity is in accordance with the finding of Dennell (1958b) that inhibition of tyrosinase by injection of phenylthiourea into mature larvae of the fly, *Calliphora vomitoria*, did not modify puparium formation. Dennell (1958a) has been led to the view that the dihydroxyphenol formed in the blood of insects by the specific action of tyrosinase on tyrosine is not of primary importance in giving rise to a tanning quinone. He thinks this activity is of importance only in the formation of melanin, although he admits the possibility that some dopa formed in the blood may be hydroxylated in the cuticle to yield a slight supply of the tanning quinone in addition to that produced by the major source. Dennell thinks the major source of the tanning quinone arises from non-enzymatic hydroxylation of phenylalanine and tyrosine to hydroquinone, which is converted to para-benzoquinone which tans the cuticle. Malek (1957) also observed that sclerotization and melanization are two separate processes in the desert locust.

Ohnishi (1954a) found that ebony larvae of *D. melanogaster,* in which the pupa case is lighter than in the wild type, showed less tyrosinase activity than did the wild type. He also found (1954b) in larvae of ebony *D. virilis,* in which the pupa case is darker than in the wild type, that tyrosinase activity was higher than in the wild type. Ohnishi (1953) also found tyrosinase activity in *D. melanogaster* larvae increases five to six hours before puparium formation, that is, nearly at the critical period of pupation hormone. These observations lead Ohnishi to the view that tyrosinase is responsible for the color of the pupae. However, Danneel (1943) found no difference in tyrosinase activity among ebony, yellow, black, and wild type *D. melanogaster,* but he did note varietal differences. Karlson and Schmid (1955) found a metamorphosis hormone in Calliphora larvae to cause browning of the cuticle, and this browning was said to be due to tyrosinase, which the authors extracted. Much additional work is needed before this question of the role of tyrosinase and its genetic control in insect metabolism can be elucidated.

SUMMARY

1. Colorimetric determinations of dopa-oxidizing activity in newly emerged adults and late larvae close to pupation time showed adult *Drosophila melanogaster* females to have higher activity rates than adult males. This may also be true for larvae, but the difference here was slight. The larvae showed higher rates than adults.

2. Larvae kept four hours at 43% relative humidity (R. H.) showed higher rates of dopa-oxidizing activity than did those kept at 100% R. H.

3. Contrary to what was expected, larvae kept at 100% R. H. pupated at a faster rate than those kept at 43%.

4. Female larvae and adults were found to be heavier than males.

5. The postulated role of tyrosinase in puparium formation is discussed.

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AN EXPERIMENTAL DEMONSTRATION OF ECHO-LOCATION BEHAVIOR IN THE PORPOISE, TURSIOPS TRUNCATUS (MONTAGU)[1]

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Many people have suspected that cetaceans use echo-location as a means of navigation. A considerable body of evidence has been marshalled on the subject, some of which shows that this is actually the case.

First, field observations made by persons trying to catch these remakable animals have shown that, not only are they far more difficult to catch than one might suppose, but also that they can avoid nets or blind channels when vision, as we know it, would seem to be ruled out (McBride, 1956; Norris and Prescott, in press).

Second, a variety of sounds are known to be produced by cetaceans, in particular by odontocetes, including some of extremely high frequencies (Wood, 1954). Further, certain of these sounds are regularly emitted when these animals locate objects in test situations where vision was kept to a minimum (see for example, Schevill and Lawrence, 1956, and Kellogg, 1958).

Third, the cetacean middle and inner ear, and the acoustic portions of the brain have been shown to be capable of the reception, binaural isolation and interpretation of high frequency sounds such as might be used in echo-location behavior (Haan, 1957; Fraser and Purves, 1959). In fact, this acoustic equipment is probably second to none in the animal kingdom, including that of the Microchiroptera.

Fourth, observations of behavior and concurrent acoustic recordings have been made using bottlenose dolphins, usually held in extremely turbid tanks, which showed that these animals were able to find their way among obstacles with little trouble, even on dark nights. Taken together these demonstrations indicate echo-location behavior with a reasonable degree of assurance, particularly since trains of high-frequency sound bursts were usually demonstrated to be emitted by the animals during solution of these problems. In none of these experimental studies was sight completely eliminated. Though water in the test tanks was extremely turbid, and human sight would not have served for more than 10 to 30 inches, still the experimenter's view of the animal was necessarily poor and it is not inconceivable that an unknown factor or factors might sometimes have directed it until sight could have played a part. For example, acute sound localization could have produced much of the behavior that has been noted. It is quite conceivable that when a test animal hears the sound of a reward fish splashing into the water, it is able to orient to the splash, swim toward the sound source, and navigate the last few feet or inches by sight.

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This sort of explanation, however, will not explain behavior in which the porpoise quickly located a reward, time after time, which was silently presented at randomized locations beneath the water's surface. It will not explain the simple experiment in which water droplets were released onto the surface of the muddy water from a height, causing immediate orientation of the porpoise but no searching behavior, after similar tests with BB shot, rocks and fish caused both orientation and searching behavior (Kellogg, 1958).

Likewise, vision seems to have been ruled out in the size discrimination test in which a bottlenose porpoise was required to choose between two species of fish of different sizes, one of which was acceptable as food and the other inacceptable. The choice was considered to be made when the test animal touched its snout to one of the target fish. The tank water was so murky that human divers could not make the discrimination when at a point equal to the distance from the porpoise's eye to the tip of its snout (Kellogg, 1959).

Since the study presented here is an extension of these previous experimental efforts, the most important ones will be described briefly in chronological order. Kellogg and Kohler (1952) transmitted sounds into a clear display tank at Marine Studios, Florida, and were able to note variations of swimming behavior that indicated hearing by captive bottlenose porpoises up to a frequency of about 80 kc. However, it seems doubtful that these observations defined upper limits for hearing since the reliability of their transducer at these high frequencies has been questioned (Schevill and Lawrence, 1953), and since it is probable that the behavior elicited by the sounds was a fright response. While the animals may have heard higher frequencies than 80 kc they need not necessarily have been frightened by them. The work was important, however, because it was suggestive of hearing capability more than four times as high as the upper limit for the average human. A more comprehensive report by Kellogg (1953) demonstrated a similar response from three separate groups of bottlenose porpoises. The upper sound level at which the response was produced 100% of the time varied from 50 to 80 kc.

Kellogg, Kohler and Morris (1953) studied the frequency spectrum of a porpoise click or pulse. They found that the majority of energy was concentrated between 20 and 30 kc. An interesting discussion of the possible utility of these sounds as echo-location signals is included. They note that such sounds become highly directional at higher frequency levels. The shorter wave-lengths also would permit greater resolution than lower pitched signals, as well as being above the majority of background noise in the sea.

Schevill and Lawrence (1953) used the conditioned response technique to determine an upper frequency limit for hearing in *Tursiops truncatus*. Their captive, held in a murky pond, was required to come to their hands for a reward upon hearing a sound signal generated in the tank. The response dropped from 100% success at 120 kc to 13% at 153 kc, the upper limit of their sound generator.

In spite of hours of listening and recording sounds, these authors reported that their test animal was able to navigate without difficulty around its tank, when not feeding, apparently without emission of any sounds at all. However, they stated that the background noise level of their test tank was "deplorably high," primarily from snapping shrimp living in the bottom mud.

In a later study these same authors (Schevill and Lawrence, 1956) reported on observations made in a quiet pond in which they found that the porpoise emitted (p. 4) "a characteristic sequence of 'creaks' as he came in for a fish and that in the last meter or so these sounds were matched to 'horizontal movements of his head.'" These sounds contained both audible and high frequency components and were associated with food location by the animal. The sound intensity was thought to be low enough so that these clicks could have been masked by the shrimp in the previous study. More significant still was the demonstration that the porpoise could locate a reward fish slipped into the water at randomized positions on either side of a net stretched in the tank. These tests represent the first experimental demonstrations of the presence of echo-location behavior in the species *Tursiops truncatus.*

Kellogg (1958, 1959) performed an extensive series of tests that provide additional evidence for echo-location behavior in the bottlenose porpoise. His test animals were able to discriminate between two gates, one closed by a plexiglass sheet, and the other open, presented in a randomized fashion. The animals invariably went through the open gate. His animals were also able to navigate through a maze of poles hung in the turbid tank, pluck a fish from an open window while ignoring one held behind glass, and as has been discussed before, distinguish between an object dropped into the water, as opposed to a measured amount of water dropped on the surface, and discriminate between different sizes of fishes suspended in the water.

Heel (1959) investigated the directional hearing capability of the harbor porpoise, *Phocoena phocoena.* He found a minimum or threshold angle of discrimination between two 6-kc tone generators to be 8° on either side of the sagittal plane of the animal's head. This figure increased somewhat with a lowering of the frequency of the signal. He states that these results are comparable with those found for humans, considering the almost five-fold increase in the speed of sound in water as compared to that in air.

Vincent (1960) has studied the sound emissions of the Atlantic saddle-back or common dolphin (*Delphinus delphis*). Three kinds of sounds are reported: whistles, popping or crackling sounds, and mewings. The popping sounds proved quite similar to the echo-location signals of *Tursiops truncatus.* Vincent was unable to record sounds above about 15 kc because of the limitations of his equipment so the entire frequency range covered by the sounds remains unknown.

The classical test of complete suppression of vision while leaving the sound emission and reception equipment intact still needed to be performed. If in this test the animal retained its ability to navigate and locate objects, we could then conclude that sight was not essential to orientation and navigation in this animal. Finally, if either the sound-sending or -receiving apparatus could be impaired, and if disorientation resulted, still in the absence of vision, then we could conclude that sound was essential to navigation, and hence that echo-location behavior did indeed occur.

We set out to attempt these tests at Marineland of the Pacific during July of 1959. Our tests continued until February, 1960, when the test animal was required for show purposes and our study had to be terminated. Since there is little likelihood of an early resumption of these experiments, some incomplete tests are reported here and are of qualitative value only.

Materials and Methods

Our experimental subject, a young adult female Atlantic bottlenose porpoise of the species *Tursiops truncatus*, was kept in a circular reinforced concrete tank 35 feet in diameter and $5\frac{1}{2}$ feet deep. Our first attempt was to train this animal to take a blindfold that would completely eliminate her vision while leaving hearing and sound transmission unimpaired.

Training

The porpoise, named Kathy, was slightly trained at the time we obtained her for our tests. Our initial step was to teach her to take cut fish rather than whole ones. This allowed us to demand a much greater number of responses for a given weight of food than could be obtained with whole fish. Thoughout the tests her diet consisted of fresh frozen Pacific mackerel (*Pneumatophorus diego*), fortified periodically with crude aureomycin and complex vitamin capsules. She was given from 12 to 15 pounds per day, depending upon her condition and drive.

Once Kathy began taking cut fish readily, she was trained to take the pieces of fish from our hands. Then we required her to allow us to touch the tip of her rostrum with the fingers of one hand before the reward was given with the other.

Throughout the tests a bridging stimulus was used to provide immediate reinforcement of desirable reactions. This consisted of a blast from a police whistle given as soon after the performance of a desired behavior pattern as possible. She soon learned that when she heard this whistle she had done what was required and would then receive her reward. In this manner we were able to reinforce actions performed across the tank or far out of reach in mid-tank.

Our plan was to use the process of multiple approximations in simulating the placement of a blindfold, step by step, until the actual act was performed. It developed that Kathy was very easily disturbed by any object that touched the areas around her eyes and blowhole, and that these approximations could be made only after many halting steps.

The first approximation was to slide one hand down her rostrum, step by step, until the fingers of one hand finally rested over one eye. In about two weeks she allowed us to hold one cupped hand over one eye for a few seconds without objection. The second approximation consisted of working both hands together into this same position, thus covering both eyes.

Once this was accomplished it was decided to approximate the blindfold itself by wearing cotton gloves during the eye-covering operation. This proved to be one of the most difficult steps in the training procedure. The animal panicked at the touch of the foreign material and training had to be redone, nearly anew. Retraining after such a balk by the animal was, however, much more rapid than when first attempted.

The first actual blindfold that was contemplated was a pair of eye cups, to be held in place by an overhead spring-steel loop, which would exert a moderate and

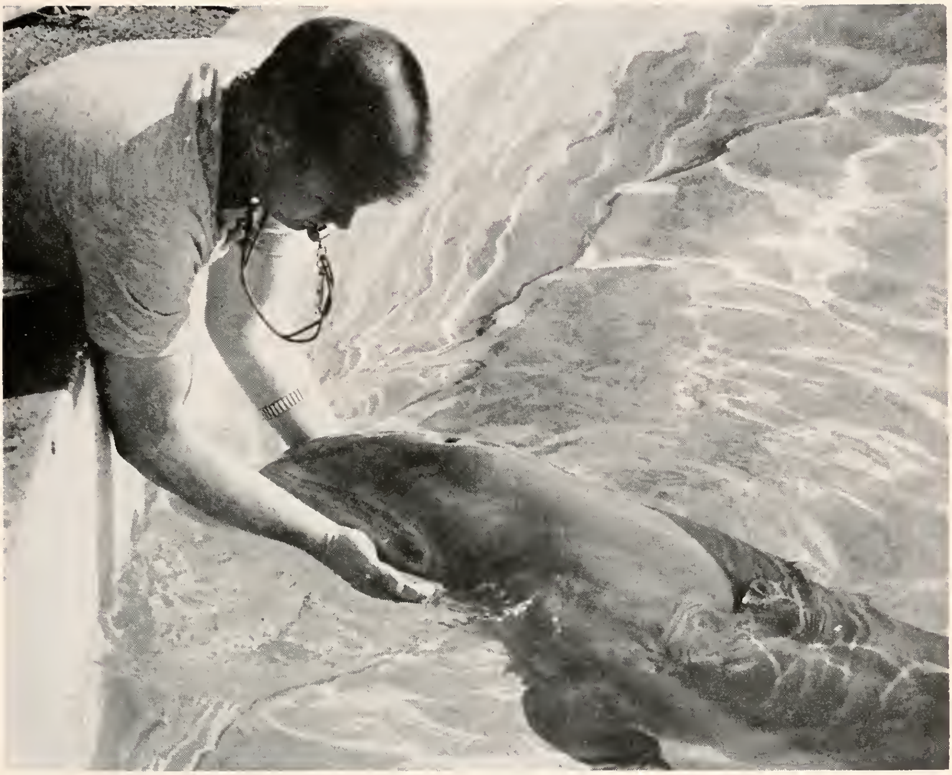

FIGURE 1. Latex suction cups being applied over the porpoise's eyes. The animal has been given a signal to come to the operator and to station herself in front of him. Note that she closes her eyes before the cups are pressed in place.

steady pressure. As an approximation of this type of blindfold, a heavy loop of tygon tubing was slipped into the gloves as far as the thumbs, curving between the operator's hands. The animal objected rather strenuously to this approximation, fleeing at first at the slightest touch of any of the tube in the region of her blowhole. Once she became reasonably accustomed to this treatment, a series of actual blindfolds built on this principle were tried, all without significant success. The animal refused to breathe while these were in place, and quickly shook them off with violent movements of her body.

After two months' work we finally stopped attempts with this type of blindfold, both because of the sensitivity of the animal to the overhead loop and because we thought it possible that the loop might cause turbulence in the vicinity of the ears and the nasal sac system that could produce erroneous results. Instead, we decided to construct a pair of latex suction cups that would just cover the animal's eyes, leaving the ear region undisturbed, and which would be without the overhead loop. This was done and after a number of trials with different cup designs a pair was developed that stayed on quite well during normal swimming movements (Fig. 1).

In the first test with these cups in place the animal swam from the operator's hands and circled the tank without any hesitation whatsoever. Never, during this or subsequent tests with the blindfolds in place, did Kathy show any tendency to bump into any obstacle in the tank. Her swimming was normal, both as to speed and maneuverability. The only detectable differences from her swimming with sight were fairly regular "rapid peering" movements with her head and neck, when she wished to "inspect" some part of her enclosure. This sort of sound-scanning has been described by both Schevill and Lawrence (1953, 1956) and Kellogg (1958, 1959).

The usual routine when testing the animal was as follows: The operator slapped the palm of his hand against the tank wall above the water line several times in succession. Kathy learned that this was her cue to come to him for placement of the blindfolds. The operator then placed his hands in the water

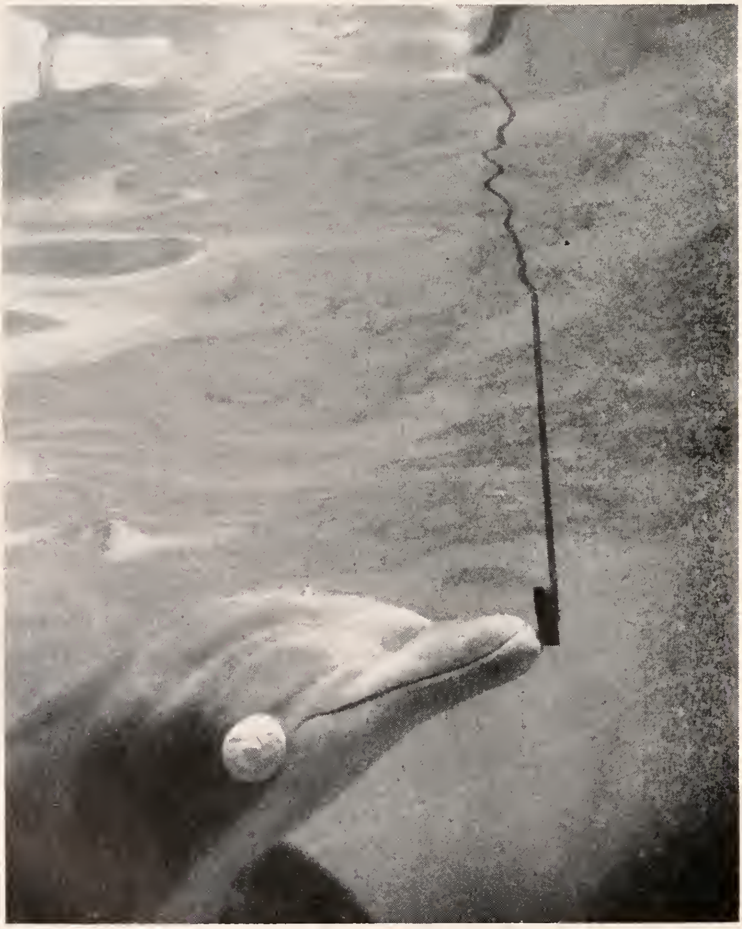

FIGURE 2. The porpoise pressing the bell target with the tip of her rostrum. Photograph courtesy of Richard Hewett.

with the cups held in his palms. She swam between his hands and permitted him to press the cups over her eyes. She always closed her eyes just before the cups were applied (Fig. 1). Then she was cued to perform the specific act required of her, by a variety of sound cues. In the case of the major test two bursts from an 18-kc subsurface transducer cued her to swim around the tank and locate a target suspended below the water surface from a randomly chosen location on the tank rim, swim to it, press it with the tip of her rostrum, thus ringing a bell, and return to the operator for a reward (Fig. 2). When recordings of her sound emission were made the reward was generally drifted in the water at the desired distance from the hydrophone. In this way she could be made to run in on the hydrophone while attempting discrimination of the small reward object.

RESULTS

A detailed analysis of sounds recorded from Kathy will not be presented here. However, there are certain observations, complete in themselves, that seem pertinent to this report.

Cruising sounds

When Kathy cruised blindfolded about the tank, not searching for a specific target object, she emitted sporadic sound pulses of the type used in echo-location. As a general rule these sounds probably kept her informed about the confines of her tank and little more. These sounds varied in number from single pulses to short trains of about six pulses. The length of each pulse varied from about 1 millisecond to 1.5 milliseconds. The repetition rates of these sounds were extremely variable. We noted that these cruising sounds were emitted by Kathy even when she was *without blindfolds,* swimming in crystal-clear water. The physical characteristics of the individual bursts of sound agree well with the clicks recorded and analyzed by Kellogg, Kohler and Morris (1953).

Searching sounds

After Kathy pressed the target while blindfolded, and rang the bell, she then returned across the tank for her reward. While this was dropped in front of the hydrophone she first reacted to the initial splash as the fish fragment (usually about a 2-inch cross-section of a mackerel) hit the water, oriented toward it, and then swam directly to it and picked it up as it drifted downward. Scanning movements by the animal usually began 12 feet or more from the reward, particularly if she failed to orient properly to the initial splash. These scanning movements became marked, usually when the animal was about three feet from the reward, sometimes becoming quite extreme as she came closer. This scanning consisted of jerky movements of the head involving lateral swings through as much as 25–30° of arc, or of circular movements of the rostrum that often caused the animal to bend and rotate her neck rather acutely. These scanning movements were particularly marked if the animal was required to pick up a reward fragment drifted very close to the hydrophone. Discrimination between two such closely adjacent objects obviously required much sound scanning, but even if the two were less than an inch apart the fish usually was picked very delicately from the water without touching the hydrophone. The fact that other investigators usually have not observed such extreme searching movements may have been due to the presence of some vision at close range in their experiments. The only case in which such exaggerated movements are reported is that observed by Schevill and Lawrence (1956) when their animal temporarily became virtually blind in both eyes. They state (p. 12): "Once when he swam past a fish in the water, he worked his way back to it slowly with very exaggerated head noddings and took the fish awkwardly deep in his mouth."

Pulse repetition rate during the initial orientation of the porpoise to her reward was variable but usually fairly low. The lowest rate observed at this time was 16 pulses per second. As the animal approached the fish this rate increased in a semi-systematic way, as a series of trains of sounds, usually reaching about 190 pulses per second just before the reward was taken. Then, the rate usually decreased abruptly to 26–50 pulses per second just as the fish was taken. Then as Kathy swerved away from the hydrophone and the wall with the fish in her mouth she often gave out a burst of pulses at extremely high repetition rates, the maximum observed being 416 per second.

Reverberations

During tests in which Kathy was required to locate a reward dropped in front of a submerged hydrophone, our recordings show as many as seven reverberations of each of her outgoing pulses between each two pulses in a train of sounds. These reverberations of her signal came from the surface, the bottom, the walls and objects suspended in the tank. In spite of this cacophony Kathy was able to locate the fish fragment and to swim directly to it. No attempt was made to play her own signals back to her.

Directionality of the sound beam

We noted that when the porpoise approached the fish fragment reward and began scanning movements with her head we began to hear marked fluctuations in the volume of the audible signal from our amplifying system.[2] This same phenomenon was also noted by Schevill and Lawrence (1956). Such directionality of the sound has been demonstrated for bats (see Griffin, 1958, p. 104). These changes in sound intensity could be correlated visually with movements of Kathy's head. When she pointed her head slightly downward so that her melon and the dorsal surface of her rostrum were in a direct line with the hydrophone, the volume increased. When she raised her snout so that the ventral surface was on a direct line with the hydrophone, the volume decreased markedly. A diver submerged in the tank with Kathy when she was blindfolded reported that the intensity of her audible signal was greatest when her rostrum was pointed directly at him, and decreased when she swung her head to the side.

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[2] During most of these experiments the sound pick-up and recording system used consisted of a U. S. Navy Model AN/PQM-1A sonar set, which covers a frequency band of 30 cycles through 33 kilocycles. The hydrophone, which contains its own preamplifier, was periodically calibrated during the tests. The system is omnidirectional and although push-button filters are an integral part of the receiver, for purposes of this study all signals were recorded in the open position.

A simple test was performed to obtain some idea of the configuration of this putative sound beam. This test consisted simply of drifting pieces of fish around the blindfolded porpoise, in various positions relative to her head, at the same time the reward signal was given. Even though Kathy was stationed virtually motionless in the water in front of the operator when these tests were made, the sound pick-up system revealed that she was emitting characteristic echo-location sounds.

Briefly, the results of these tests were that she was able to detect and snap up any fish that descended from above and ahead of her melon, while she invariably lost those that drifted below the level of her rostrum, even though they were sometimes within less than an inch of her jaws (Fig. 3). Her only successes in obtaining reward fragments drifted below her jaws came when, during her scanning movements, she bent far enough downward so that the fish was in line with the horizontal axis of her rostrum, or above it.

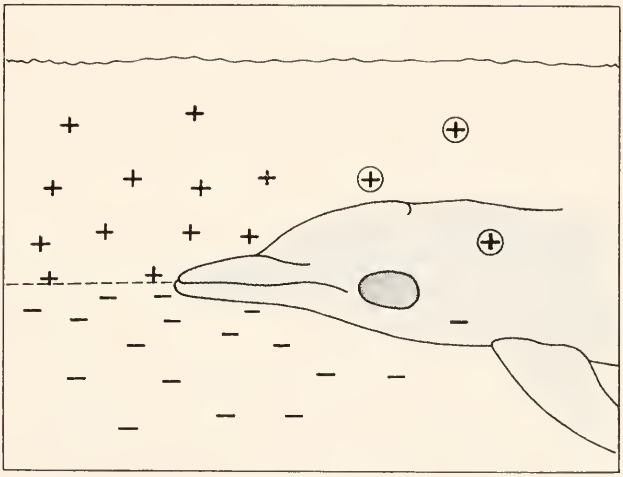

FIGURE 3. A diagram showing the detection of fragments of fish drifted into the water near the blindfolded porpoise. Plusses indicate the positions where food fragments were detected immediately and snapped up without hesitation. Minuses indicate positions at which reward fragments were lost. Circled plusses indicate positions at which the porpoise did not immediately snap up the reward, but returned and recovered it on another pass.

Fish drifted lateral to her head were picked up as far back as her eye, as long as they were above the level of her jaws. Fish drifted down on top of the anterior apex of her melon were located with no trouble. Fish drifted down above her head in the vicinity of her blowhole were usually picked up when she turned and made a second pass at them as they drifted toward the tank bottom.

Thus it appears that the animal probably sends, and may also receive, sound in a directional fashion during echo-location. It also appears that the site of this directionality is localized, at least in part, in the region of the upper jaw and melon. The observations by the diver, and those of the authors who could correlate sound intensity with the position of Kathy's head in relation to the hydrophone, indicate that the upper jaw and melon region is connected with beamed sound transmission though they do not preclude the possibility of sound reception at the same site. The echo-location system of the bottlenose porpoise therefore does not appear to function solely on the basis of an omnidirectional sound field emanating from a localized or point source as has been assumed in the past. In view of the wide range of frequencies present in individual sound pulses it seems likely that the degree of directionality may vary systematically with frequency. One bit of evidence gained during our experiments tends to support this view. A small especially-constructed hydrophone, sensitive to a narrow band of frequencies centered around 100 kc, was lowered into the tank. The only time signals from this hydrophone could be picked up on our oscilloscope was when Kathy pointed her rostrum accurately at the hydrophone.

Experimental interference with the sound-sending and -receiving equipment

The lack of dependence upon vision is amply demonstrated by the blindfold experiment. While the sound emission and associated behavior is strong evidence for echo-location in the blindfolded animal, the crucial test is still disruption of the sound path of the blindfolded porpoise in some way, coupled with the results of such disruption. The first approach to this problem was simply to cover the external ear openings with a patch of material that would disrupt the passage of sound. In this case two small suction cups were used, each 1 inch in diameter and lined with $\frac{1}{8}$ of an inch of foam neoprene sheeting. Such foam neoprene forms an excellent acoustic mismatch with water because of its multitude of tiny trapped gas bubbles. The porpoise did not object to having these cups pressed in place over her external ear openings, and then navigated perfectly with both eye and ear cups in place. This result is, however, considered inconclusive for two reasons. First, it was impossible to tell how firmly the neoprene liner of the cups was pressed against the skin of the animal at the periphery of the neoprene patch. Conceivably there could have been places in which the barrier consisted of no more than the latex of the cup and the thin border of the neoprene patch. Thus there would have been no effective sound barrier. Second, if odontocetes rely upon transmission of sounds through the blubber and flesh to the tympanum and middle ear ossicles, as has been recently postulated (Haan, 1957), no disorientation would have been expected. According to this theory the external auditory canal is a rudiment. Support for this idea is provided by the discovery that in at least one species of porpoise (*Phocoenoides dalli*) the canal does not reach the surface of the skin (Norris and Prescott, in press).

Because of these uncertainties we turned to another approach. A mask of $\frac{1}{4}''$ neoprene sheeting was constructed that covered Kathy's entire melon and upper jaw, including its tip. We hoped to train the animal to wear this mask while blindfolded. If the sound beam, presumed to emanate from this region, was actually essential to her blind navigation she should then manifest disorientation in some way when wearing this mask. As a means of attachment the mask was equipped with latex suction cups at appropriate points and was held on her jaws by means of rubber bands. Although Kathy was relatively docile and continued to come to her trainer upon command, we were never successful in making her wear this mask for more than a few seconds. On the few occasions when it was attached properly to her head and jaws, she backed away from the trainer out into the open water of the tank and shook her head violently until the mask was shaken loose. Then, in a spirit of cooperation with her trainers, she sometimes picked up the floating mask and carried it back to them. This friendly but adamant refusal to wear the mask continued for two months until the termination of our tests. During this time she gave not the slightest hint that she would eventually submit to wearing the mask. During this entire time she never swam forward with the mask in place.

While her behavior during these mask tests did not give clear-cut evidence of disorientation it may be that the refusal we observed was the only kind of behavior we could expect from a highly intelligent animal faced with the loss of her visual and auditory sensory windows.

The discrimination ability of a blindfolded porpoise

Several tests, all incomplete, were performed to test the refinement of Kathy's echo-location ability.

First, two lines on pulleys were stretched across Kathy's pool, 10 feet apart From each of these lines one-inch diameter iron pipes were suspended at four- to seven-foot intervals. Each pipe reached nearly to the bottom of the tank. Since the lines could be moved independently, a maze of variable characteristics could

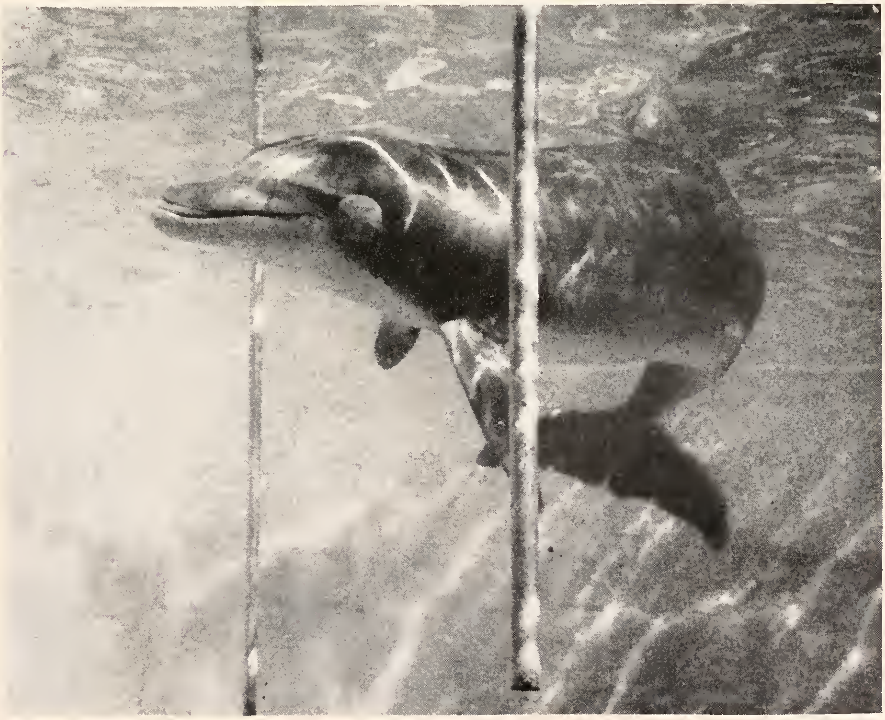

FIGURE 4. The porpoise maneuvers between two maze poles. Photograph courtesy of Richard Hewett.

be produced. Kathy was run through this maze several times daily for a period of nearly two months, and only once did she touch a pole. Her ability to snap up a reward fish fragment dropped inches from a pole was remarkable. She would often circle the pole near which the reward had been dropped, bend her neck sharply, and delicately pluck the fish out of the water as it sank. At the same time, she avoided hitting the poles or the wall behind her with the utmost finesse, when her flukes and tail stock swung around (Fig. 4). Her one contact with a pole seemed to be a deliberate sidewise slash with her tail flukes, as she passed between two poles.

This test differed from that reported by Kellogg (1958) in that the poles of the maze were smaller (one inch in diameter as opposed to triangular poles two inches on a side), and because the poles were often much closer together. The poles in Kellogg's maze were eight feet apart.

The ability of porpoises to discriminate between objects of different sizes by sound has been shown by Kellogg (1959). It seems probable from the following test that discrimination is not limited to size differences alone but extends to objects of the same size but of different sound-reflecting characteristics.

A $1\frac{1}{2}''$ long veterinary gelatine capsule ($\frac{1}{2}$ ounce capacity) was filled with water and carefully weighted with small pebbles so that it would sink slowly when placed in water. Then a piece of mackerel flesh was cut the same size as the capsule. When both were thrown simultaneously into the tank and the reward whistle signal sounded, blindfolded Kathy approached both slowly sinking objects, scanned both with swings of her head, and without hesitation snapped up the mackerel fragment. This test was repeated several times with the same results. She never gave the sinking capsule more than a passing inspection. It should be noted that the mackerel fragment was cut entirely from muscle and skin and did not contain portions of the swim bladder or other gas filled spaces.

Discussion

This demonstration of navigation and discrimination by a blindfolded bottlenose porpoise provides a tool for the study of cetacean echo-location that, if used further, should allow direct attack on several of the most vexing problems involved in this navigation system. In particular, refined work on thresholds of discrimination by sound should now be relatively simple. Also, it should be possible to define accurately the characteristics of the sound field emitted by a cetacean, using a trained blindfolded animal. Perhaps such a trained animal could be used to explain the most obscure problem of all—that of the actual mechanism by which the porpoise uses trains of sound pulses to "inspect" its environment.

Summary and Conclusions

1. A method was devised whereby an Atlantic bottlenose porpoise could be blindfolded with soft latex suction cups. This method has allowed some analysis of the echo-location system of this animal, and should provide a tool for further study.

2. The blindfolded animal was remarkably adept at obstacle avoidance and in the location of small food fragments.

3. The blindfolded porpoise proved able to discriminate reward fragments from water-filled capsules of the same size, weighted to sink at the same rate as the fish. She was also able to locate and pick up fish fragments drifted within an inch of obstacles such as a hydrophone or maze poles, picking the food out of the water with the utmost delicacy, and without touching the nearby obstacles.

4. During the porpoise's approach to a reward fragment the animal emitted sounds characteristic of echo-location in the species. These were trains of short pulses whose repetition rate increased semi-systematically as the animal came nearer. Pulse repetition rates varied from a low of 16/second at the beginning of a run, to about 190/second near the fish. Then a brief lull period was usually observed just before the reward was taken, when repetition rate dropped to 26–50/second. After the fish was taken the rate sometimes rose in a "brush of pulses" to as high as 416/second. Individual pulses varied in length from 1 to 1.5 milliseconds.

5. Sporadic echo-location sounds were emitted by the animal when simply cruising around her tank. These sounds were emitted even when the animal was not blindfolded, swimming in clear water.

6. Some evidence is presented that indicates the use of a beam or cone of sound emanating from the forehead region of the animal, above the level of the mouth.

7. Attempts were made to interfere with the sound path of the blindfolded animal, to determine if disorientation would result. The first attempt, that of placing acoustically opaque suction cups over the external ear openings of the animal failed to interfere, in any visible way, with the behavior of the animal. This failure is thought to be due either to inadequate blockage of sound by the cups, or to sound conduction by adjacent blubber and muscle.

The second attempt was to train the subject to wear an acoustically opaque mask over her upper jaw and forehead region. In spite of two months' intensive training the animal refused to wear the mask for more than a few seconds. Even then she only wore it long enough to back away from the operator into open water and there to shake it off. She refused completely to swim forward with it in place. This refusal is suggestive that her sound beaming apparatus was being interfered with by the mask.

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SKELETON FORMATION OF SEA URCHIN LARVAE. III. SIMILARITY OF EFFECT OF LOW CALCIUM AND HIGH MAGNESIUM ON SPICULE FORMATION

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It has been known for some time that a deficiency of calcium, magnesium or sulfate ions arrests spicule formation in sea urchin larvae (Pouchet and Chabry, 1889; Herbst, 1897, 1904). On the other hand, to the writer's knowledge, the effects of an excess of these ions have hardly been reported except with respect to calcium (Okazaki, 1956, 1960; Bevelander and Nakahara, 1960). For this reason, exposure to an excess of magnesium was tested and found to cause the same abnormality of the spicules as that previously shown to result from a deficiency of calcium ions (Okazaki, 1956).

I. Effects of Serial Changes of Calcium or Magnesium Concentration

Early developmental stages of *Hemicentrotus pulcherrimus* were used as the experimental material. Low-calcium media were prepared by mixing calcium-free sea water (containing the normal amount of magnesium) and natural sea water in 6:4, 8:2, 9:1 and 19:1 ratios. As high-magnesium media, solutions of twice and four times the magnesium concentration of natural sea water (both with the normal amount of calcium) were prepared in the way shown in Table I, and these solutions were mixed in 3:1 and 1:1 ratios. The resultant media will be referred to as 0.4 Ca, 0.2 Ca, 0.1 Ca, 0.05 Ca, 2 Mg, 2.5 Mg, 3 Mg and 4 Mg; these values refer to the concentration of calcium or magnesium relative to that of natural sea water.

Table I

Volume ratios of stock solutions for preparing high magnesium media (2 Mg, 4 Mg)

Natural S. W.	Stock solutions					Resultant media
	$M/3$ $MgCl_2$	$M/3$ $CaCl_2$	$M/2$ KCl	$M/3$ Na_2SO_4	$M/2$ $NaHCO_3$	
762.0	208.0	6.5	4.3	18.2	1.0	2 Mg
285.0	618.0	21.0	14.0	58.0	4.0	4 Mg

Transfer of the larvae into the experimental media was done at either of two stages: early gastrula stage with calcareous granules, or mid-gastrula stage with small triradiate spicules. The subsequent formation of the spicules in 2 Mg, 2.5 Mg, 3 Mg and 4 Mg corresponded respectively to that in 0.4 Ca, 0.2 Ca, 0.1 Ca or 0.05 Ca. The characteristics of the spicular shape in each pair of calcium-low and magnesium-high media were as follows:

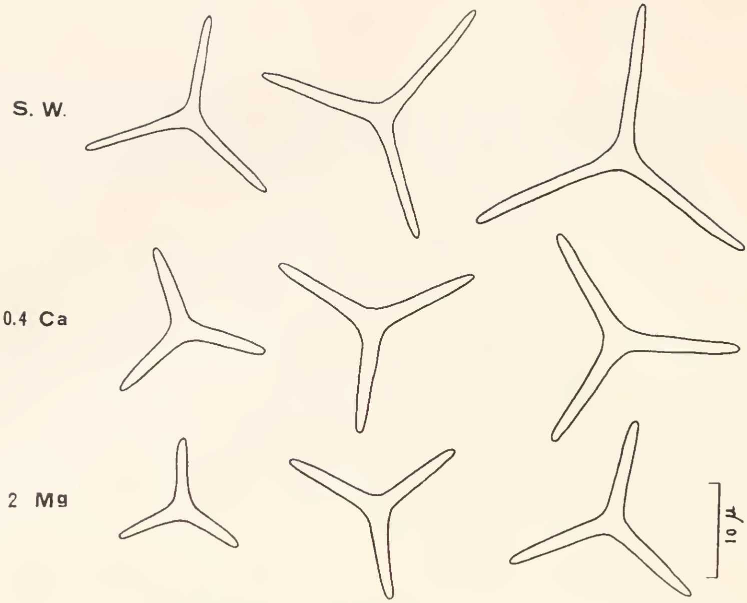
S. W.
0.4 Ca
2 Mg
10 μ

Figure 1. Spicules of *Hemicentrotus pulcherrimus* larvae, transferred to 0.4 Ca and 2 Mg at early gastrula stage with calcareous granules, sketched 7 hours later (13° C.).

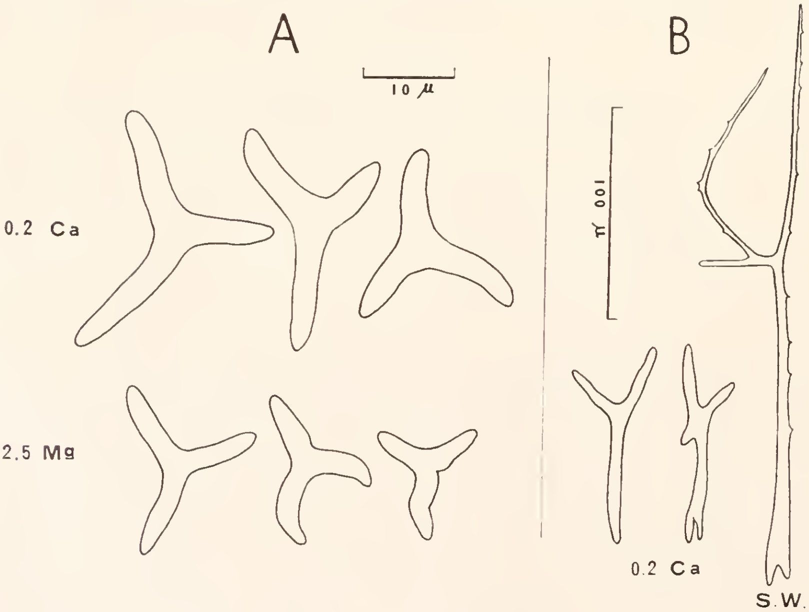
A
B
10 μ
100 μ
0.2 Ca
2.5 Mg
0.2 Ca
S.W.

Figure 2. Spicules of *H. pulcherrimus* developed from calcareous granules in 0.2 Ca and 2.5 Mg. A, sketched 22 hours after transfer at 13° C. (early pluteus stage of controls); B, after 50 hours.

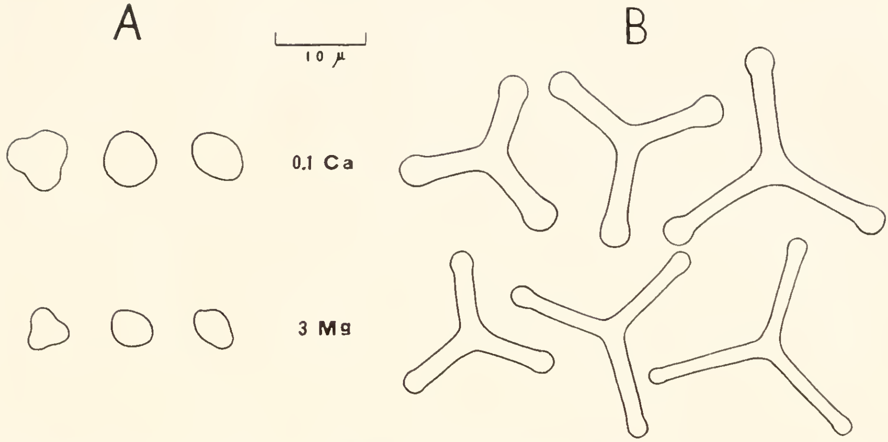

FIGURE 3. Spicules of *H. pulcherrimus* in 0.1 Ca and 3 Mg. A, transferred at calcareous granule stage and sketched 25 hours later (13° C.); B, transferred at triradiate spicule stage and sketched 23 hours later at 13° C. (compare with Figure 1).

(1) *0.4 Ca and 2 Mg.* The calcareous granules of the early gastrula acquire a triradiate form, although lengthening of the spicule is more or less retarded while its thickening is accelerated (Fig. 1). These triradiate spicules ultimately develop so that they have all the component rods of the pluteus skeleton (Fig. 2, B), which are seemingly normal but shorter and thicker than those of the sea water control. This is true even if the treatment is begun after the spicule has taken a triradiate form in sea water. On the whole, spicular growth in 0.4 Ca is superior to that in 2 Mg, but the ratios, width/length of the spicule, are much the same in these two media.

(2) *0.2 Ca and 2.5 Mg.* In comparison with the spicules found in 0.2 Ca, those in 2.5 Mg are smaller in size but quite similar in shape. Since lengthening of the spicule is strikingly inhibited, while its thickening is not so much impeded,

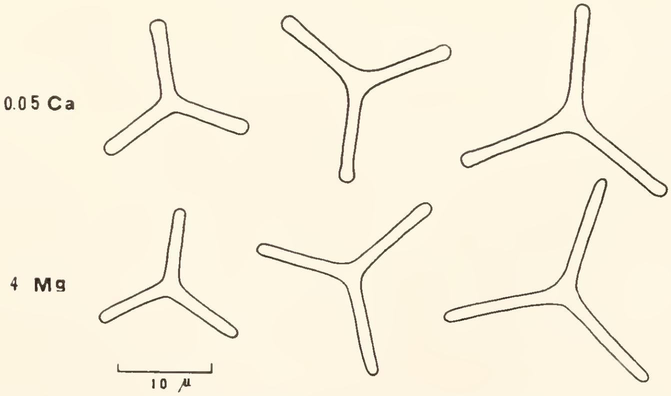

FIGURE 4. Spicules of *H. pulcherrimus* thickened in 0.05 Ca and 4 Mg. Twenty-five hours after transfer (13° C.).

the spicules become stockier than those in the previous pair of solutions. Although the calcareous granules eventually acquire three arms by the time the controls reach the early pluteus stage, they are somewhat abnormal in that the lengths of the arms and the angles between them are not uniform (Fig. 2, A). On the next day, the spicules in these media only grow to trifurcate skeletal rods when the controls develop to four-armed plutei (Fig. 2, B).

(3) *0.1 Ca and 3 Mg.* Spicules only thicken without elongating, the thickening being faster in 0.1 Ca than in 3 Mg. In these media, calcareous granules increase their masses only as spherical bodies (Fig. 3, A), and triradiate spicules, if transferred, form a spherical mass at each tip of their arms (Fig. 3, B).

(4) *0.05 Ca and 4 Mg.* Spicules thicken without increasing in length, in the same manner as in 0.1 Ca or 3 Mg, although the rates of thickening are slower (compare Fig. 3, B and Fig. 4).

II. WHICH IS EFFECTIVE, CONCENTRATION (OF CALCIUM OR MAGNESIUM) OR RATIO (CALCIUM/MAGNESIUM)?

In the calcium series, the calcium concentration was reduced while the magnesium level was kept constant; in the magnesium series, the magnesium concentration was increased while the calcium level was kept fixed. This produced a continuous reduction in the calcium/magnesium ratio through the two series, leaving room for the suspicion that the similarity in the effect of low calcium and high magnesium may be due rather to a decrease in the ratio between the concentrations of the two ions than to the change of concentration, *per se*, of the respective ions. To clarify this point, the following four series of experiments were run, in which the values of calcium/magnesium were varied by mixing calcium-magnesium-free sea water, $M/3$ $MgCl_2$, $M/3$ $CaCl_2$, $M/2$ KCl and $M/3$ Na_2SO_4 in appropriate ratios. The concentrations of Mg^{++}, Ca^{++}, K^+ and SO_4^{--}

TABLE II

Ca and Mg levels of the experimental media

Series 1	Ca	10/10	4/10	2/10	1/10
	Mg	10/10			
Series 2	Ca	10/10	4/10	2/10	1/10
	Mg	5/10			
Series 3	Ca	10/10	4/10	2/10	1/10
	Mg	1/10			
Series 4	Ca	10/10	4/10	2/10	1/10
	Mg	10/10	4/10	2/10	1/10

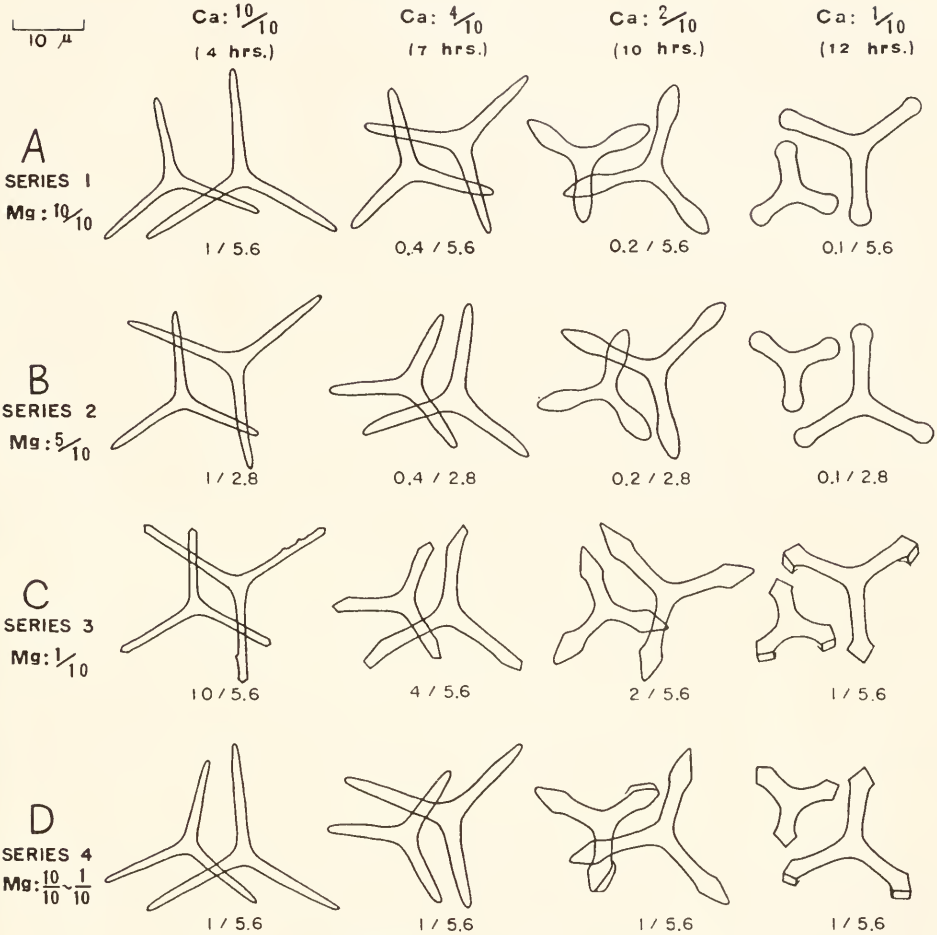
10 μ
Ca: 10/10 (4 hrs.)
Ca: 4/10 (7 hrs.)
Ca: 2/10 (10 hrs.)
Ca: 1/10 (12 hrs.)
A
SERIES 1
Mg: 10/10
1 / 5.6
0.4 / 5.6
0.2 / 5.6
0.1 / 5.6
B
SERIES 2
Mg: 5/10
1 / 2.8
0.4 / 2.8
0.2 / 2.8
0.1 / 2.8
C
SERIES 3
Mg: 1/10
10 / 5.6
4 / 5.6
2 / 5.6
1 / 5.6
D
SERIES 4
Mg: 10/10 ~ 1/10
1 / 5.6
1 / 5.6
1 / 5.6
1 / 5.6

FIGURE 5. Correlation of width/length of the spicule with calcium concentration (regardless of calcium/magnesium). Calcium and magnesium concentrations expressed by fractions of normal amounts (*cf.* Table II). Fraction at bottom of each figure indicates calcium/magnesium in medium. Numerals in parentheses indicate hours after transfer.

ions were calculated on the basis that respective amounts in natural sea water are 5.6/100, 1/100, 1/100 and 2.9/100 *M*.

In each series, the concentration of calcium was changed to 10/10, 4/10, 2/10 and 1/10 that of natural sea water, potassium and sulfate being kept at the normal level. The concentration of magnesium, on the other hand, was varied among the four series; it was maintained at the normal amount in Series 1, reduced to 1/2 in Series 2 and to 1/10 in Series 3, while in Series 4, the magnesium was added in a gradient (10/10, 4/10, 2/10 and 1/10) similar to that of the calcium concentration (see Table II). The pH of each resultant medium was adjusted to 8.2 by bicarbonate buffer. Series 1 is the same as the calcium-low media used in Part 1, the left member of the series containing 10/10 calcium and 10/10 magnesium, which correspond to natural sea water in composition.

Gastrulae with small triradiate spicules were transferred to these media and after appropriate exposure, the shapes of the spicules in Series 2–4 were compared with those of Series 1.

The results are summarized in Figure 5. No difference was discernible between the spicules in Series 2 and those of Series 1 (compare Fig. 5, B with 5, A), although the calcium/magnesium ratio of the former is twice that of the latter medium. In series 3, the calcium/magnesium values were increased above the normal level, in contrast to Series 1. Notwithstanding this, the width/length of the spicule increased with a decrease in the calcium concentration to the same degree as in Series 1, although the spicular tips became square as the result of the specific influence of magnesium deficiency (compare Fig. 5, C with 5, A), the details of which will be reported in a later paper. In Series 4, the calcium/magnesium value remains the same as that of sea water. However, in 0.4 Ca-Mg (in which the calcium and magnesium concentrations were simultaneously reduced to 4/10 the normal level), the spicules were the same in all respects as those in 0.4 Ca. In 0.2 and 0.1 Ca-Mg, although the characteristic effect of magnesium deficiency was evident at the tips of the spicules as in Series 3, spicular width/length was much the same as in the corresponding medium of Series 1 (compare Fig. 5, D with 5, A).

CONCLUSION

These observations indicate that (a) calcium deficiency and magnesium excess in the culture medium have the same effects on spicular form, and (b) the effects are due to the absolute values of the concentrations of these ions and not to the ratios between them. This result may find an easy explanation on the basis that the magnesium ions must be competing with the calcium ions and replacing them, although they are not adequate substitutes in performing the physiological function of calcium (*cf.* Heilbrunn, 1952, pp. 538, 598).

The writer wishes to express her gratitude to Prof. K. Dan and Dr. J. C. Dan for their kind criticism and advice for the work. Her thanks are also due to the staff of the Misaki Marine Biological Station for use of the Station's research facilities.

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THE UPTAKE AND TURNOVER OF S^{35} SULFATE BY LEBISTES

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The uptake of calcium-45 and strontium-90 from the water in which they swim is linear with time for fresh-water fishes (Rosenthal, 1956, 1957), and the linear relationship is valid for the carcass, muscle, viscera and spine. The linear uptake also appears to be valid for the uptake of P^{32} phosphate and Cl^{36} chloride by brown trout (Phillips *et al.*, 1958), and for Cr^{51} chromium ion in brook trout (Knoll and Fromm, 1960). However, the rate of incorporation of calcium-45 and strontium-90 appears to be a function of the concentration of the nuclide in the water, which is adequately described by a function of the form $Y = aX^b$ where Y and X refer to rate of incorporation (Y) and water concentration (X), respectively, and a and b are constants. This relationship has recently been confirmed for strontium ion in the euryhaline *Tilapia* by Townsley *et al.* (1958–59).

The alkaline earth elements are bone-seeking elements and once incorporated into bone may remain for many days. Sulfur metabolism is also intimately associated with bone growth and development (Dziewiatkowski, 1953) but sulfate is not considered a "bone-seeker." In conjunction with other studies concerning the metabolism of mineral elements in various fishes, it was of interest to study the sulfate anion in the same manner as previously reported for calcium-45 and strontium-90 in *Lebistes* (Rosenthal, 1956–60).

MATERIALS AND METHODS

Adult male wild type guppies, averaging 100 mg. in weight, were obtained from commercial sources. All fish were fed daily and maintained with commercial dry food supplemented with frozen adult brine shrimp twice weekly. Ten to fifteen fish were placed in 500 ml. artificial pond water in wide-mouth glass bowls containing carrier-free S^{35} sulfate obtained from the Oak Ridge National Laboratories. The basic solution (Prosser *et al.*, 1945), adjusted to pH 7.0, contained 0.5 mM/liter each of the following analytical reagent grade salts: sodium chloride, potassium nitrate, and magnesium chloride, and 0.2 mM/liter calcium chloride. For equilibration studies, 50–80 adult male *Lebistes* were placed in 4 liters of pond water contained in plastic bags with a surface area of 400 square inches for 7 days before the experiments were started. Additions of varying amounts of sodium sulfate were made in place of sodium chloride whenever possible.

Turnover studies were performed by placing 50–80 fish in 1500 ml. aged tap water containing S^{35} sulfate in plastic bags with a surface area of 400 square inches. After 7 days' uptake, the fish were transferred to plastic bags containing 4 liters of non-radioactive aged tap water. The fish were transferred to new water daily for the first three days and every third day thereafter until completion of the experiment. The local tap water averaged 110 ppm sulfate (range 84–167 ppm) during the period of this study.

Samples of the carcass and tissues were dissolved in nitric acid as previously described (Rosenthal, 1956), and 0.1–ml. aliquot portions were plated on lens paper circles in stainless steel planchets. After drying under a heat lamp the samples were assayed for radioactivity in a windowless gas flow counter to a probable error of less than 5%. Under these conditions, self absorption of the sample and lens paper was a constant and all samples were corrected for self absorption and decay. The efficiency of the counter for S^{35} sulfate was 3.2×10^9 cpm/millicurie. The contribution of the isotope to the total sulfate concentration was negligible and was not considered in the calculations.

RESULTS

As shown in Figure 1, male *Lebistes* placed in artificial pond water containing 20 ppm sulfate take up radioactive sulfate in a linear fashion during a ten-day experimental period, and the linear relationship is not affected by changes in the specific activity of S^{35} sulfate present in the water. However, the relationship between the rate of uptake of S^{35} sulfate and the activity of the water in which the fish swim at a given non-radioactive sulfate concentration is adequately de-

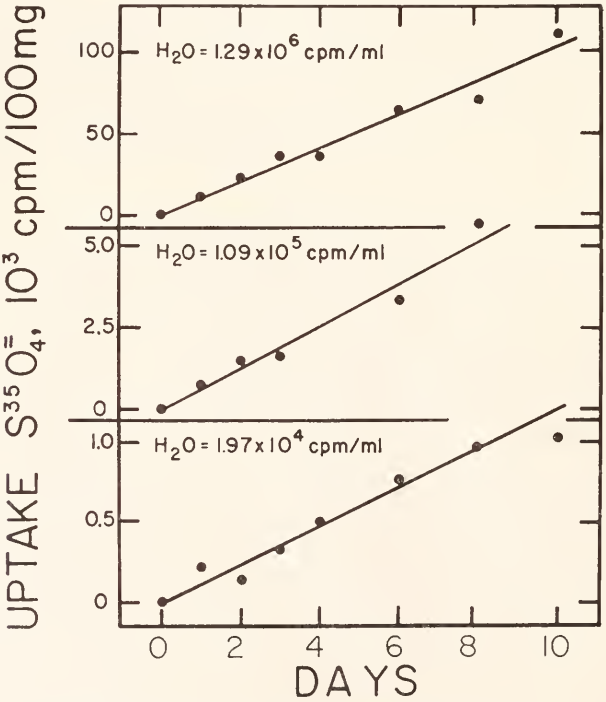

FIGURE 1. The uptake of S^{35} sulfate versus days in artificial pond water containing 20 ppm non-radioactive sulfate. Each point represents average data obtained for 4 to 8 fish.

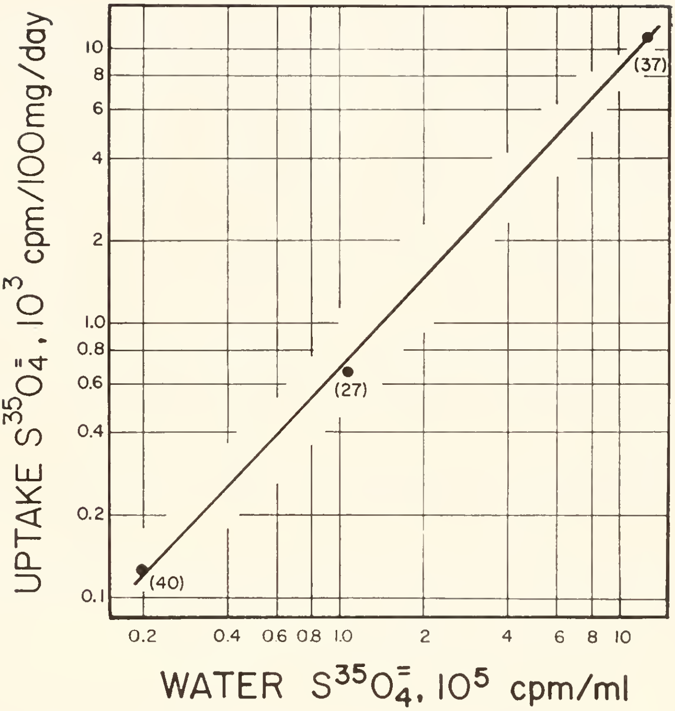

FIGURE 2. The rate of uptake of S^{35} sulfate versus the S^{35} activity of artificial pond water containing 20 ppm non-radioactive sulfate. Each point represents average data obtained for 27 to 40 animals.

scribed by a logarithmic equation of the form $Y = aX^b$, as shown in Figure 2. The rate of uptake of S^{35} sulfate is markedly affected by the non-radioactive sulfate concentration of the water (Fig. 3A). In this experiment, the uptake of S^{35} sulfate from water containing 1 ppm non-radioactive sulfate was linear for about 6 days. Between 6 to 10 days, equilibrium was established between the body of the fish and the external environment, and no further S^{35} sulfate accumulated. At concentrations between 48 ppm and 192 ppm non-radioactive sulfate, the rate of uptake of S^{35} sulfate progressively decreased as the sulfate concentration increased, and the uptake deviated from linearity after 2 to 4 days. These data indicate that the animals were essentially saturated with sulfate and the uptake of S^{35} sulfate rapidly approached equilibrium with the body sulfate pool. This interpretation was substantiated when the data were found to be adequately described by a logarithmic function (Fig. 3B).

In other experiments, the uptake of S^{35} sulfate from water of varying non-radioactive sulfate concentrations was also determined. In these experiments the fish were sacrificed 5 days after being placed in the radioactive water. It is apparent that maximal accumulation occurs when S^{35} sulfate is taken up from water containing from 1 to 20 ppm sulfate (Fig. 4). At sulfate concentrations between 20 and 192 ppm, the uptake of S^{35} sulfate decreases rapidly as the sulfate concen-

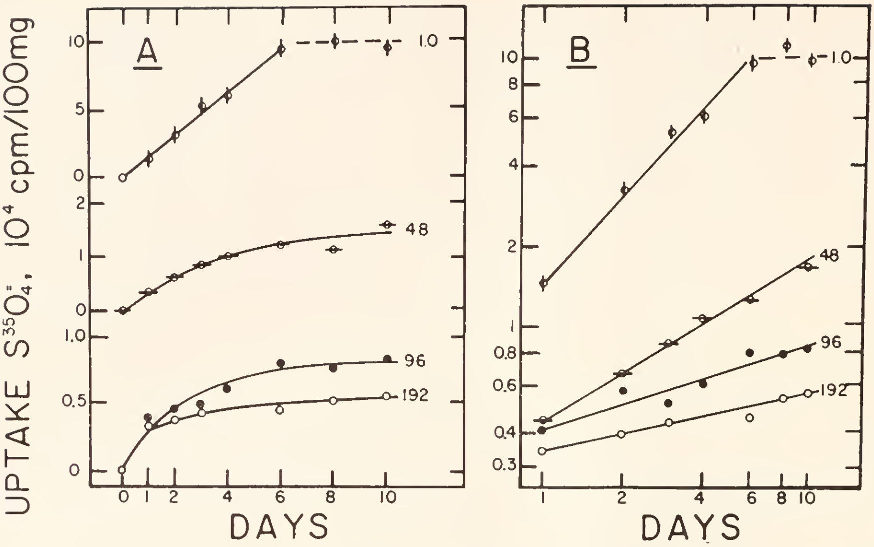

FIGURE 3. The uptake of S^{35} sulfate versus days in artificial pond water containing 3.2×10^5 cpm/ml. S^{35} sulfate. Each point represents average data obtained for 4 to 6 fish. A. Data plotted arithmetically. B. Data plotted logarithmically.

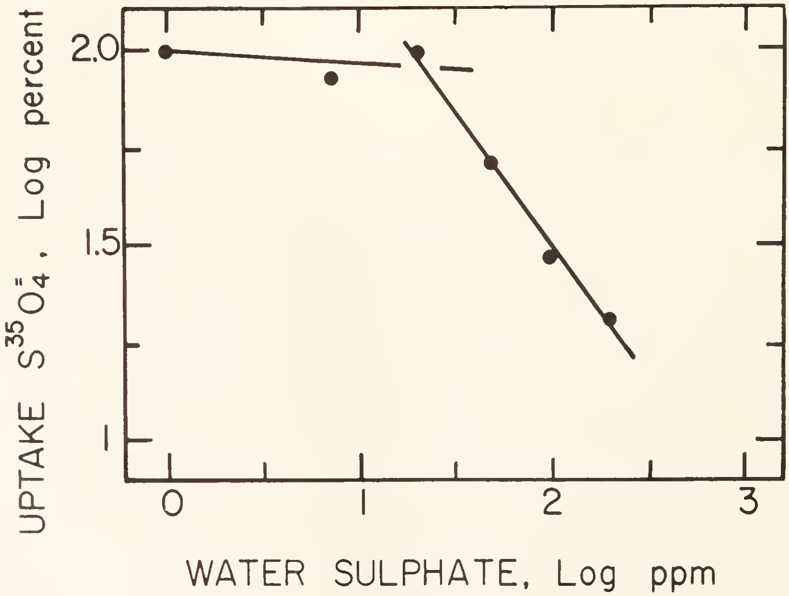

FIGURE 4. The rate of uptake of S^{35} sulfate versus concentration of non-radioactive sulfate plotted as the logarithms. Each point represents average values obtained for 3 to 6 animals. The water contained 3.7×10^5 cpm/ml.

tration increases, and the accumulation of S^{35} sulfate is logarithmically related to the sulfate concentration of the water.

That the rate of uptake of S^{35} sulfate from the water in which the fish swim is markedly affected by the body sulfate pool of the animal is shown in Figure 5. Male *Lebistes* were equilibrated for 7 days in artificial pond water containing 15 ppm sulfate. After this period of sulfate depletion, the fish were divided into three groups and transferred to bowls containing artificial pond water prepared to contain 15 ppm, 75 ppm and 200 ppm non-radioactive sulfate in addition to S^{35} sulfate. Other experiments were performed in which the fish were equilibrated in artificial pond water containing 75 ppm and 200 ppm non-radioactive sulfate before the animals were subjected to the experimental conditions.

The uptake of S^{35} sulfate by *Lebistes* after equilibration at 15 ppm appears to be non-linear when the isotope is taken up from water containing 15 ppm non-radioactive sulfate ion. The data are adequately expressed by an exponential equation of the form $Y = ae^{kX}$, thus indicating altered sulfate metabolism in these animals. However, the sulfate-deficient animals take up S^{35} sulfate linearly from water containing 75 ppm and 200 ppm non-radioactive sulfate.

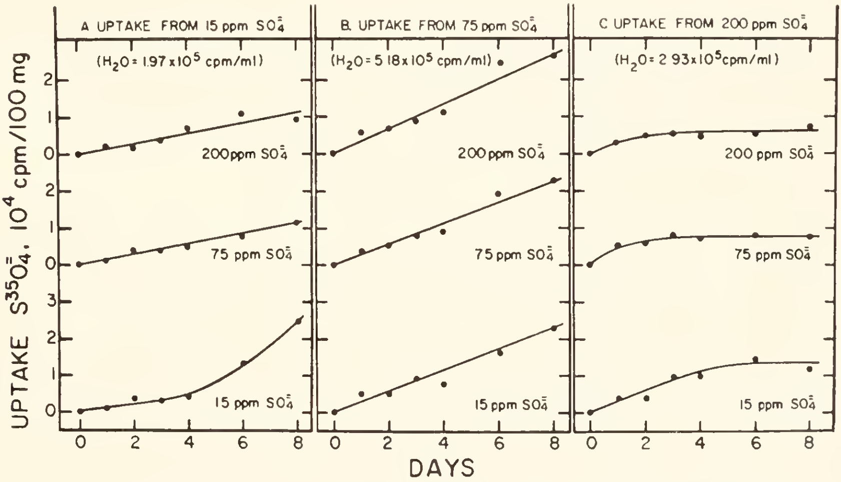

FIGURE 5. The uptake of S^{35} sulfate versus days in artificial pond water at various non-radioactive sulfate concentrations. The sulfate concentration for equilibration is given below each curve. Each point represents average values obtained for 4 to 7 animals.

Following equilibration at 200 ppm non-radioactive sulfate, the uptake of S^{35} sulfate reaches maximum incorporation within 2 to 3 days. This finding indicates that the sulfate stores of the animals were fully saturated and the body sulfate pool rapidly approached equilibrium with the external medium. Under all other experimental conditions, the uptake of S^{35} sulfate from the water in which the fish swim was linear during the experimental period.

The incorporation of S^{35} taken up from the water in which the fish swim is linear during a six-day experimental period for all tissues of the body that were studied (Fig. 6). The concentration of radioactivity is greatest in the soft tissues

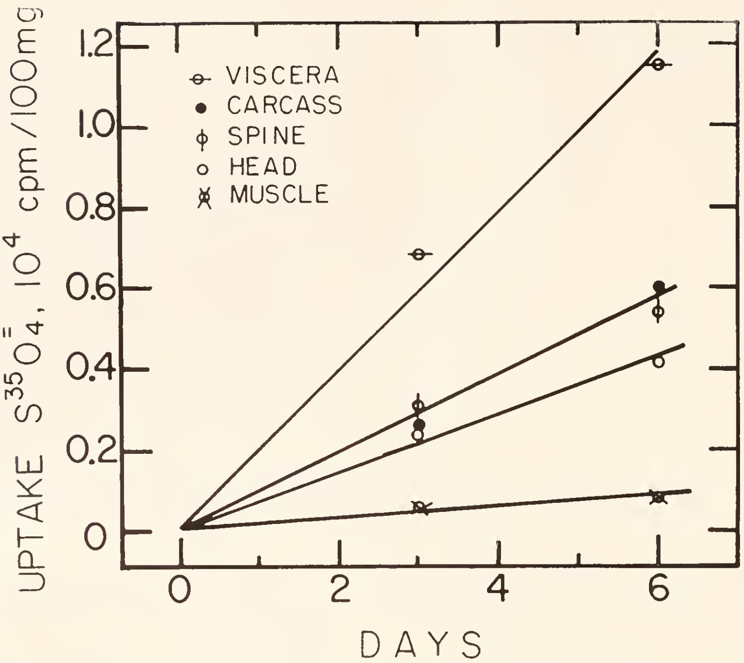

FIGURE 6. The incorporation of S^{35} sulfate into carcass and tissues versus days in artificial pond water containing 75 ppm non-radioactive sulfate. Each point represents average data obtained for 3 to 8 fish. The water contained 2.2×10^5 cpm/ml.

of the visceral organs (including the tissues of the gastrointestinal tract) while muscle incorporates the least amount and bony tissues, such as spine and head, incorporate intermediate amounts (Table I). When the data are calculated as the per cent of the total body radioactivity in the organs that were studied (Table I), 50% of the total body activity could be accounted for. The remaining 50% must have been present in the skin, scales and fins which constitute the unanalyzed remainder.

TABLE I

*Tissue distribution of S^{35} taken up from water****

Organ	$\left(\frac{\text{Organ weight}}{\text{Body weight}}\right) \times 100$	$\left(\frac{\text{cpm/organ}}{\text{cpm/total body}}\right) \times 100$	$\left(\frac{\text{cpm/100 mg. tissue}}{\text{cpm/100 mg. body}}\right)$
Body	100	100	1.00
Head	20.25 ± 0.35	13.28 ± 1.49	0.66 ± 0.07
Viscera	13.12 ± 0.35	27.89 ± 3.22	2.44 ± 0.38
Spine	2.57 ± 0.08	2.35 ± 0.36	0.91 ± 0.12
Muscle*	40.00	6.28 ± 0.30	0.16 ± 0.02
Remainder**	24.06 ± 0.54	50.20 ± 4.60	2.10 ± 0.19

* Muscle tissue is assumed to represent 40 per cent of the wet weight of the body.

** Calculated by difference.

*** Each value represents average data obtained for 25–28 fish ± standard error of the mean.

The rate of turnover of S^{35} sulfate incorporated into body tissues was determined in fish during a 35-day experimental period. The loss of radioactivity from the body could be resolved into three well defined components (Fig. 7) with biological half-lives of 1.4 days for the most rapidly disappearing component, 10.3 days for the second component and at least 600 days for the third component. The various tissues lost radioactivity with varying rates which ranged from very rapid for viscera to very slow for muscle. The disappearance curve for muscle was logarithmic during the entire experimental period, and was found to have a biological half-life of 660 days.

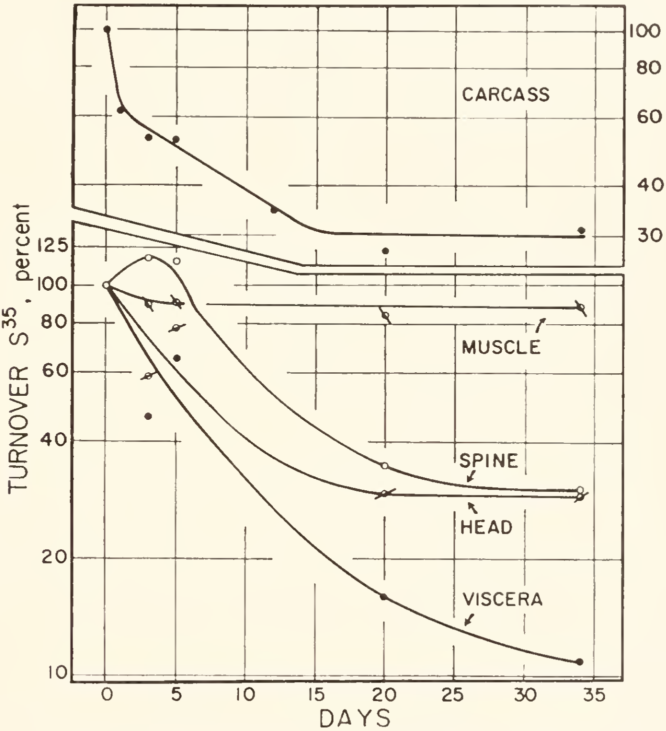

FIGURE 7. The disappearance of incorporated S^{35} sulfate from the carcass and tissues of *Lebistes* versus time in non-radioactive water. The fish initially contained 1.0×10^4 cpm/100 mg. body wt. Each point represents average data for 4 to 7 fish.

DISCUSSION

It is apparent from these data that male *Lebistes* accumulate S^{35} sulfate from the water in which they swim in a linear fashion under specific conditions of sulfate ion concentration of the external environment. When the animals are depleted of sulfate, the rate of uptake departs from linearity when the isotope is taken up from water containing low (15 ppm) sulfate concentration. In animals saturated with sulfate by equilibration in water containing 200 ppm sulfate, the uptake of S^{35} sulfate rapidly approaches an equilibrium. The uptake of S^{35} sulfate depends, therefore, on the physiological state of the animal and presumably on many other factors that require further study. In general, the optimum water sulfate concentration for these animals appears to be somewhat greater than 75 ppm, under the ionic and osmolar conditions of the artificial pond water used in these studies in order to maintain the sulfate integrity of the animal and to supply sufficient sulfate ion for growth and development.

The rate of uptake of S^{35} sulfate into the total carcass of *Lebistes*, and the logarithmic relationship between the rate of uptake of the isotope and the isotope concentration of the water, are very similar to that found for calcium-45 and strontium-90 in these fish (Rosenthal, 1957). Since fresh-water fishes are not considered to actively drink water (Knoll and Fromm, 1960; Reid *et al.*, 1959), the gastrointestinal tract probably plays only a minor role for the absorption of the ions. This leaves only the gills and other epithelial integuments (skin, etc.) as the regulatory tissues, although ion transport through the skin may vary with the species. In *Tilapia*, epithelial membranes seem to be permeable to calcium and strontium (Reid *et al.*, 1959) but tuna skin is not permeable to these ions (Chipman, 1956). Independent of the specific tissue involved in the accumulation of inorganic ions in *Lebistes*, the regulatory system transporting anions and cations from the water into the body and tissues of the animal appears to be the same. This conclusion was also reached for brown trout by Phillips *et al.* (1958) and for *Tilapia* by Townsley *et al.* (1958–59).

The accumulation of S^{35} in the various organs and tissues of the body may be considered to fall into three main categories. The greatest accumulation and the fastest turnover time occur in the viscera. The body, spine and head (which also includes the gills) fall into an intermediate group while muscle represents the third group with lowest concentration of S^{35} and the longest half-life. The very slow turnover rate of S^{35} in muscle ($T^{\frac{1}{2}} = 660$ days) is similar to that previously found for strontium-90 (Rosenthal, 1957) but is longer than that found for calcium-45 (Rosenthal, 1956).

A comparison of the disappearance curves for the total body and the various organs and tissues indicates that the two most rapidly disappearing components represent a loss of radioactivity from the combined viscera, spine, head and unanalyzed "remainder" while the slowest disappearance curve most probably represents the disappearance of radioactivity primarily from muscle tissue and to a lesser extent from the tissues of the spine and head. The rapid initial loss of radioactivity by the spine and head during the first 20 days indicates little, if any, sequestration of S^{35} into mineral components of these tissues. Although the turnover rate of S^{35} sulfate by tissues of the head and spine can not be adequately determined in a short term experiment of 34 days, examination of Figure 7 indicates a biological half-life ranging between 400-600 days, as determined during the last 15 days of the experiment. This value is an estimate at best and requires further study for more definitive values. Since sulfate ion is rapidly incorporated into sulfated polysaccharides of the ground substance in rats (Dziewiatkowski, 1952), the similarity of the biological half-lives for the body, spine, and muscle may represent the turnover time of this substance.

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CONCLUSIONS

1. The uptake of S^{35} sulfate from the water in which the fish swim is linear with time for male *Lebistes* under specific conditions of water composition, but the uptake is modified in fishes depleted or saturated with sulfate. The rate of uptake of S^{35} sulfate is logarithmically related to the activity of the isotope in water. These results are similar to that found for calcium-45 and strontium-90, and suggest that the ion transport regulatory system is similar for all ions studied.

2. The turnover time of S^{35} sulfate for most tissues approximates a biological half-life of 600 days and presumably represents the turnover time of chondroitin sulfate and other sulfated polysaccharides.

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THE PRESENCE OF DECAPOD-PIGMENT-ACTIVATING SUBSTANCES IN THE CENTRAL NERVOUS SYSTEM OF REPRESENTATIVE CIRRIPEDIA[1]

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The comparative distribution among the arthropods of chromatophore-activating substances has been studied by several investigators and has been reviewed recently by Carlisle and Knowles (1959). Chromatophorotropins have been shown to be present in crude extracts of the nervous systems of various malacostracans, namely representatives of the Isopoda, Natantia, Reptantia and Stomatopoda. These materials are also extractable from the corpora cardiaca of the cockroach, *Periplaneta* (Brown and Meglitsch, 1940) and from the nervous system of the horseshoe crab, *Limulus* (Brown and Cunningham, 1941).

Chromatophorotropins which play a role in the normal adaptive pigmentary responses of the Crustacea (Fingerman, Sandeen and Lowe, 1959) are believed to originate in neurosecretory cells of the nervous systems. The first study of the correlation between the distribution of a particular type of neurosecretory cell in the nervous system and the chromatophorotropic activity of extracts of portions of the nervous system was done using the crab, *Sesarma* (Enami, 1951a, 1951b). This subject is also reviewed by Carlisle and Knowles (1959).

Neurosecretory cells have been shown to be present in crustaceans lower phylogenetically than the Malacostraca. Lochhead and Resner (1958) have described their occurrence in the branchipod, *Artemia,* and Barnes and Gonor (1958) have described them in the Cirripedia, including *Pollicipes polymerus, Chthamalus dalli, Balanus glandula, B. hesperius laevidomus, B. nubilis,* and *B. rostratus.* No previous attempt has been successful, however, in finding chromatophorotropic activity in nervous system extracts of barnacles.

A chromatophorotropin which disperses the black pigment of *Uca pugilator* is one of the more widely distributed principles. Abramowitz (1937) has shown it to be present in extracts of the eyestalks of the prawn, *Palaemonetes,* the shrimp, *Crago,* as well as in extracts of the eyestalks of *Uca.* The most extensive survey of its presence was made by Brown (1940). In this study differential amounts of black-pigment-dispersing activity were found in extracts of eyestalks in the Brachyura, *Uca, Carcinus, Callinectes* and *Libinia,* in the Natantia, *Crago* and *Palaemonetes* and in the anomuran, *Pagurus.* In this same study the distribution of a substance which concentrates *Palaemonetes* red pigment was described. These two materials were found to exist in various proportions in eyestalks of the different crustaceans that were studied. The *Uca* black-dispersing substance is one of the chromatophorotropins that was found in *Limulus* (Brown and Cunningham, 1941). Neither these authors nor Snyder-Cooper (1938) found *Palaemonetes* red-concentrating material in extracts of the nervous system of *Limulus*.

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Few interspecific tests have been made for retinal-pigment-activating substances. The probability that the light-adapting hormone is a substance distinct from the chromatophorotropins is discussed by Carlisle and Knowles (1959). Both distal pigment light-adapting and dark-adapting substances are present in extracts of eyestalks and portions of the nervous system of *Palaemonetes* (Kleinholz, 1935; Brown, Hines and Fingerman, 1952; Fingerman, Lowe and Sundararaj, 1959).

Several aspects of the life-cycle and physiology of the barnacle, *Balanus improvisus,* have been studied at the Duke Marine Laboratory (Costlow and Bookhout, 1953, 1957). As a first step in learning something about the endocrinology of the Cirripedia the following studies were undertaken in the hope of finding some homologies with other crustaceans. The fiddler crab, *Uca* and the prawn, *Palaemonetes*, about which so much is known, are common members of the fauna of this region and were selected as suitable test animals for interspecific studies.

Materials and Methods

The barnacles used in these studies were collected as needed from three different ecological niches near the Duke Marine Laboratory, Beaufort, N. C. Individuals of *Balanus eburneus* were taken from the surfaces of pilings at low tide; individuals of *Chelonibia patula,* from the carapaces of blue crabs taken in crab pots. The individuals of *Lepas* sp. were collected from a piece of driftwood carried by the currents into the vicinity of the laboratory. The fiddler crabs, *Uca pugilator*, were collected at low tide behind the laboratory buildings on Piver's Island, and the common prawns, *Palaemonetes vulgaris,* were collected from among the local algae at low tide. All of the animals were used within one to three days after being brought into the laboratory.

For all of the experiments the nervous systems of the barnacles, including supraesophageal ganglia, circumesophageal connectives and thoracic ganglia, were dissected under a dissecting microscope. This dissection actually involved the removal of the rest of the barnacle from the nervous system. With practice the whole system was obtained intact in a large percentage of the trials. Extreme care was taken to insure that no tissues other than nervous system were included. Nervous systems from ten barnacles were placed in a glass dish. Most of the water was removed with filter paper and the tissue was thoroughly triturated. This material was suspended in the desired amount of filtered sea water.

Twelve to twenty-four hours before a chromatophore assay for *Uca* black-dispersing substance, both eyestalks were removed from a group of fiddler crabs. This operation causes the pigment in the black chromatophores to concentrate and the pigment in the white chromatophores to disperse. Animals in this condition respond to injections of extracts of various portions of nervous systems of the same species. Black pigment will always disperse, and with some extracts white pigment will concentrate. The behavior of the yellow and red pigments was not considered in this study.

When testing the responses of the fiddler crabs the standard dose was 0.05 cc.

Using a hypodermic syringe and a 26-gauge needle, this was injected at the base of the fourth or fifth walking leg into the ventral hemocoel. An arbitrary system of designating concentration of materials was established. In keeping with the method used by Sandeen (1950) a concentration of one would be the equivalent of one nervous system in one dose. Since the stock suspension of barnacle nervous system extract was ten systems in 1.5 cc. sea water, there would be one nervous system in three doses, and the concentration of the suspension is referred to as a concentration of $\frac{1}{3}$.

In order to determine the presence of chromatophorotropins for activating the pigments of *Palaemonetes,* these prawns were prepared 12–24 hours before an experiment in one of two ways. To test for red-pigment-concentrating activity both eyestalks were removed from a group of prawns, using a sharp scalpel. The eyestalks were cauterized to prevent bleeding. As a result of this operation the red pigment becomes dispersed and remains in this condition, and is responsive to an extract which produces red pigment concentration. To test for red-pigment-dispersing activity only one eyestalk was removed from specimens of prawns. These animals have their red pigment concentrated when they are placed on a white background under laboratory lighting, and yet are more responsive to injections of extracts which produce red pigment dispersion than are normal animals (Brown, Webb and Sandeen, 1952).

For the prawns, a dose of 0.02 cc. of material was used. This was injected into the animals between two of the middle abdominal segments, a little to the side of the mid-dorsal line. For these assays the barnacle extracts were made using 10 nervous systems in 0.2 cc. of filtered sea water, giving an extract with a concentration of one.

In determining chromatophore responses of both fiddler crabs and prawns the chromatophore scale of Hogben and Slome (1931) was used. In this scale the number 5 designates fully dispersed pigment and 1, fully concentrated pigment. The numbers 4, 3 and 2 designate intermediate conditions. For each assay the recipients were injected at zero time and then, at appropriate time intervals thereafter, the average chromatophore stage for each animal was determined with the aid of a dissecting microscope. The chromatophores of the fiddler crab that were observed were those on the antero-ventral aspect of the largest segment of the second or third walking leg. In the case of the prawns the large red chromatophores in the hypodermis above the heart were used. All experiments were begun between the hours of 1 and 2 PM and concluded before 6 PM in order to be working within a limited range of any diurnal rhythm of sensitivity of the chromatophores (Webb, Bennett and Brown, 1954).

To determine the presence of *Palaemonetes* distal retinal-pigment-activating substances, one-eyed prawns were prepared 12–24 hours previous to an experiment as described above. The condition of the distal retinal pigments was determined in the manner described by Sandeen and Brown (1952) with the modifications introduced by Fingerman, Lowe and Sundararaj (1959). A dissecting microscope was equipped with an ocular micrometer, and a total magnification of approximately 60 was employed. Each prawn was held ventral surface down in a petri dish of sea water under the objective. Using an appropriate balance between transmitted and reflected light two measurements of the eye were taken: the length of the translucent area resulting from partial light adaptation at the distal edge of the eye, and the total length of the retina from the edge of the cornea to the proximal edge of the dorsal black pigment spot. The first figure divided by the second figure was used as the distal pigment index. The higher the index, the more light-adapted is the eye of the prawn.

These assay animals for distal pigment activators were kept on a black background in subdued laboratory lighting for the duration of each experiment. Under these conditions the initial condition of the distal pigment is intermediate, and if the appropriate activators are present both light-adaptation and dark-adaptation can be elicited.

Since interspecific injections were being made between Crustacea separated rather widely in their phylogenetic relationships, two additional precautions were taken. All extracts of the nervous system of the barnacles were boiled and centrifuged. The supernatant fluid, free of precipitated proteins, was used. In addition, for at least one of each type of experiment, extracts of barnacle muscle were prepared in the same manner as the extracts of nervous system and these were injected into assay animals as controls. Sea water was similarly injected into assay animals as controls for each type of experiment.

Experiments and Results

Uca black-dispersing substance

For the initial experiment each of ten eyestalkless *Uca pugilator* was injected with a dose of an extract of nervous systems of *Balanus eburneus.* As a control each of 10 similar eyestalkless *Uca pugilator* was injected with a dose of an extract of muscle of *B. eburneus.* The chromatophores of each animal were staged at 15, 30, 45, 60, 90, 120, 180, and 240 minutes following the injection. The average chromatophore stage for each group of animals for each time interval was determined and these averages were used to plot Curves A and A′ of Figure 1.

By examination of Figure 1, A, it can be seen that the extract of nervous system of the barnacle caused the black pigment of all the assay animals to become maximally dispersed (stage 5) by 45 minutes following injection. The black chromatophores remained dispersed for two hours, began to concentrate before three hours and were again concentrated after four hours. The extract of barnacle muscle produced a slight reaction in three out of ten animals. The average responses of these control animals are shown in Curve A′ of Figure 1. The white chromatophores of the fiddler crabs were affected by neither of these extracts nor by any others used in this study.

For the second experiment the goose neck barnacle, *Lepas* sp., was used. An extract of nervous systems similar to that of *Balanus* was assayed on ten eyestalkless *Uca pugilator.* One dose of filtered sea water was injected into each of six similar *U. pugilator* as a control. The chromatophores were staged at 0, 15, 30, 45, 60, 90, 120 and 150 minutes and the average value for each time was determined. These average values were used to plot Curves B and B′ in Figure 1. Curve B of Figure 1 shows that the black chromatophores of all the assay animals were maximally dispersed at 30 minutes and remained so for one hour. Concentration of pigment began between 60 and 90 minutes. At the last reading, 150 minutes, the chromatophores were still partially dispersed. By extrapolation of the curve it is apparent that the total reaction lasted at least three hours. No black pigment responses were elicited by the injection of sea water into control animals. This is shown in Curve B′ on Figure 1.

It is clear from comparison of Curves A and B of Figure 1 that there is a difference in the effectiveness of these two extracts. It seemed desirable, therefore, to compare the chromatophore responses from a series of dilutions of extracts of nervous systems of different barnacles. Since *Lepas* sp. was no longer available it was decided to use a third barnacle, *Chelonibia patula.*

Ten nervous systems of *Chelonibia patula* were dissected and extracted in 1.5 cc. sea water. As explained above the concentration of this extract was designated as one third. From this extract a series of dilutions, 1/9, 1/27, 1/81, 1/243, were prepared. In the same manner an extract of nervous systems of *Balanus eburneus* and a similar series of dilutions were prepared. Each of these extracts was assayed on five eyestalkless *Uca.* Chromatophore stages of each individual were determined at 0, 15, 30, 45, 60 minutes and each 30 minutes thereafter until the chromatophores returned to the initial condition. The average chromatophore stage of the five animals was determined for each time that a reading was taken for each solution. From these averages a total activity value for each concentration was calculated. This was done in the following way. The number 1, designating the initial stage, was subtracted from each average value. The resultant figure represents the extent of the response noted at any given time. Then all of these values for any given concentration of extract and for the duration of the response were totaled. This activity value is, therefore, a measure of the magnitude and duration of the response. These activity values as a function of the logarithm of

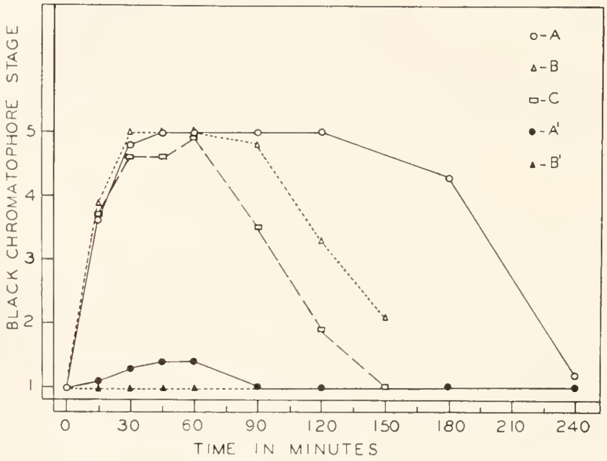

FIGURE 1. The relationships between the average black chromatophore stage of eyestalkless *Uca* and the time in minutes following the injection of extract of central nervous system of *Balanus eburneus* (A), extract of nervous system of *Lepas* sp. (B), extract of nervous system of *Chelonibia patula* (C), extract of muscle of *Balanus eburneus* (A′), sea water (B′).

dilution for both *Balanus* and *Chelonibia* are plotted in Figure 2, Curves A and B. From this figure it can be seen that a total activity of 3.6 is produced when the *Balanus* material is in a dilution of 1/243. The material from *Chelonibia*, however, produces a significant activity only up to a dilution of 1/27. Since the total activity produced by the *Balanus* extract of 1/3 concentration (24.4) is essentially the same, or even somewhat less, than that produced by the *Balanus* extract in the first experiment (25.7 for 180 minutes), it seemed wise to repeat the *Chelonibia* dilution experiment. This was done with the one modification that ten eyestalkless *Uca*, instead of five, were used as assay animals. The results were essentially the same as previously and the total activity values are plotted as a function of log of dilution as Curve C in Figure 2. It seems clear that the initial extract of nervous systems of *Chelonibia* had a concentration of active material 1/3 to 1/9 that of the *Balanus*.

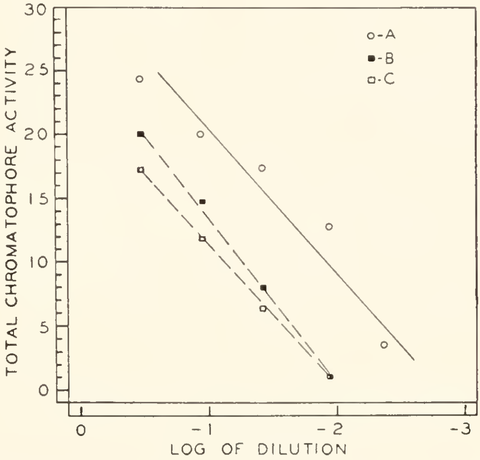

FIGURE 2. The relationships between the total chromatophore activity of the black pigment of *Uca* and the log of the dilution of extracts of nervous system of *Balanus eburneus* (A), extract of nervous system of *Chelonibia patula*, Experiment 1 (B), and extract of nervous system of *Chelonibia patula*, Experiment 2 (C).

To complete the comparison of the three genera of barnacles that were used in this study, the hourly readings of the average chromatophore stages for the nervous system extract of *Chelonibia* at 1/3 concentration from the second dilution experiment were plotted as Curve C of Figure 1. The results graphed in Figure 1 illustrate that the *Chelonibia* extract produces less chromatophore activity than the *Lepas* extract, and the *Lepas* extract produces less than the *Balanus* extract. Before any conclusion can be drawn about specific differences in this function, however, experiments would have to be designed to eliminate the possibilities (1) that there are antagonistic chromatophorotropic principles in these extracts, and (2) that the barnacles show variations as a result of their own biological activities. This is beyond the scope of the present study. It should be pointed out, however, that each barnacle extract was made initially by dissecting nervous systems from ten barnacles. This procedure was adopted in an attempt to eliminate the possibility of individual variation among barnacles. This does not, however, eliminate the possibility of fluctuations within a whole population of animals.

In keeping with the data that are being collected on the biochemical nature of crustacean chromatophorotropins an experiment was designed to determine whether the active principle from the barnacle would be inactivated by exposure to a proteolytic enzyme. An extract of nervous systems of *Balanus eburneus* was prepared as described previously. An enzyme preparation was made using a fresh supply of Matheson Coleman and Bell trypsin powder (1–100) in a concentration of 1 part per 10,000 of filtered sea water. A portion of the enzyme was inactivated by placing it in a boiling water bath for 10 minutes. Three mixtures were prepared: (1) 3/4 barnacle CNS extract plus 1/4 boiled trypsin, (2) 3/4 barnacle CNS extract plus 1/4 active trypsin, and (3) 3/4 barnacle CNS extract plus 1/4 sea water. In tube 2, therefore, the final concentration of active enzyme was 1/40,000. These three test tubes were incubated at 40° C. for 5 minutes and then immersed in a boiling water bath for 5 minutes. The tubes were centrifuged and the supernatant taken up in syringes. Each extract was assayed on three eyestalkless *Uca*. The chromatophore stages of these assay animals were determined at 0, 15, 30, 45, 60 and 90 minutes. The extract containing boiled trypsin and that containing only the barnacle substances produced the usual black-pigment-dispersing reaction and no white pigment concentration. The extract that had been exposed to the active enzyme produced no response of the *Uca* black pigment. This experiment was repeated a second time using the shorter incubation time of one minute. The same results were obtained. The chromatophorotropic principle in the barnacle extract was completely inactivated in one minute or less by a 1/40,000 solution of trypsin.

Palaemonetes chromatophorotropic principles

To test for the presence of *Palaemonetes* red-pigment-concentrating activity three separate experiments were conducted. For each of the first two experiments, extracts of CNS of *Balanus eburneus* were prepared using ten nervous systems in 0.2 cc. sea water, and as controls, extracts of a comparable amount of muscle of *B. eburneus*. Each extract was assayed on ten eyestalkless *Palaemonetes* whose chromatophores were dispersed, with the average chromatophore stage for the groups of ten animals being 4.3 to 4.7. The chromatophores were staged at 0, 5 and 15 minutes. When the appropriate activator is present a maximal response occurs within 5 minutes. No responses of these chromatophores were observed. The two experiments were averaged together and the results are shown in Figure 3, A. To ascertain the reliability of the test a third experiment was conducted. For this experiment the activity of a similar barnacle extract was compared with that of an extract known to produce red pigment concentration, *i.e.*, 24 *Palaemonetes* eyestalks ground and suspended in 0.24 cc. of sea water. Each of these extracts was assayed on ten animals. The chromatophore stages were determined at 0, 5, 15, and 30 minutes. The average chromatophore stages for each of these times were calculated and were used to plot the curves in Figure 3, B. It can be seen from this figure that the eyestalk extract produced the expected and profound concentration of the red pigment. Again the barnacle extract showed no apparent influence on the dispersed red pigment.

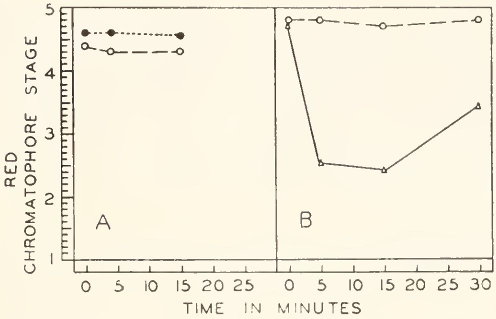

FIGURE 3. The relationship between the average stage of the red chromatophores of eyestalkless *Palaemonetes* and the time in minutes following the injection of (open circles) extract of nervous system of *Balanus eburneus;* (closed circles) extract of muscle of *Balanus eburneus;* and (open triangles) extract of eyestalks of *Palaemonetes.*

To test for the presence of *Palaemonetes* red-dispersing substance four experiments were conducted. For the first three experiments nervous system extracts of barnacles were prepared in the usual manner. Two types of control solutions were used; one was sea water and the other, an extract of supraesophageal ganglia of *Palaemonetes,* ten ganglia in 0.2 cc. of sea water. The extract of supraesophageal ganglia of *Palaemonetes* is known to produce dispersion of the red pigment of one-eyed *Palaemonetes* (Brown, Webb and Sandeen, 1952). In the fourth experiment three separate extracts of muscle of *Balanus* were used. Each of these extracts was assayed on a group of ten one-eyed *Palaemonetes.* The chromatophores were staged at 0, 15, 30, 60, 90, and 120 minutes in all except the third experiment in which the zero reading was omitted. Average values for each of the time intervals were determined for each extract.

There were, therefore, three experiments of ten animals each, for each kind of extract. Since the results were qualitatively the same for these three trials the averages for the three were calculated. These average values were used to plot the curves which are shown in Figure 4. It can be seen in Figure 4 that

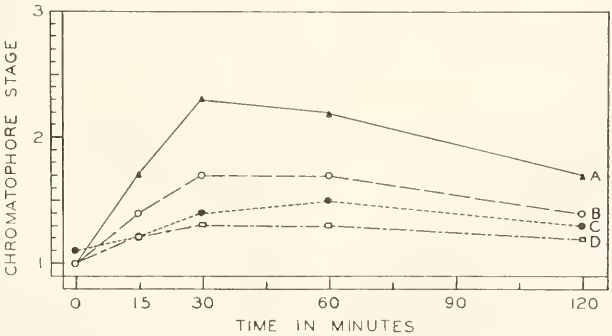

FIGURE 4. The relationship between the average stage of the red chromatophores of one-eyed *Palaemonetes* and the time in minutes following the injection of extract of supraesophageal ganglia of *Palaemonetes* (A), extract of nervous system of *Balanus* (B), extract of muscle of *Balanus* (C), sea water (D).

the extract of supraesophageal ganglia of *Palaemonetes* produced the predicted response with an average chromatophore dispersion of 2.3 at 30 minutes. Both the injection of sea water and the injection of extract of barnacle muscle produced a slight response. The extract of nervous systems of *Balanus,* however, produced a decided dispersion of the red pigment, but of less magnitude than did the extract of supraesophageal ganglia of *Palaemonetes.*

Distal retinal-pigment-activators

To test for the presence of distal retinal-pigment-activators, ten one-eyed *Palaemonetes* were placed in each of three small black-painted enamel pans for two hours before the beginning of each experiment. Before receiving an injection the distal pigment index of each animal was determined as described under Materials and Methods. The average value for each group of ten animals was calculated. Under these conditions these groups of animals had average distal pigment indices ranging from .083 to .116, indicating an intermediate degree of light adaptation.

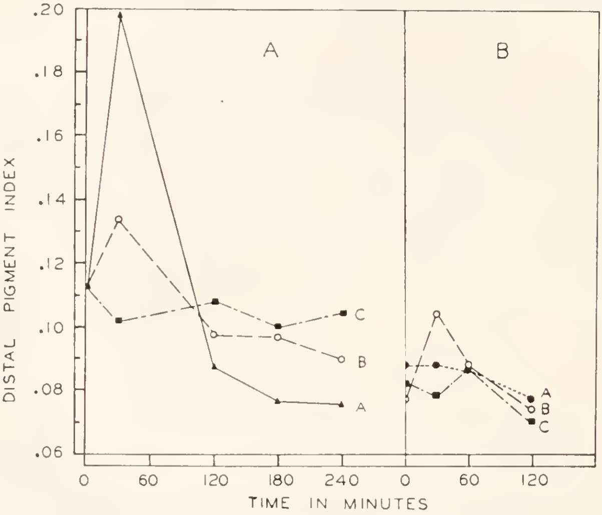

FIGURE 5. Part A. The relationship between the average distal pigment index of one-eyed *Palaemonetes* and the time in minutes following the injection of extract of eyestalks of *Palaemonetes* (A), extract of nervous system of *Balanus* (B), sea water (C). Part B, following the injection of extract of muscle of *Balanus* (A), extract of nervous system of *Balanus* (B), sea water (C).

In the search for distal-pigment-activators four experiments were performed. For the first two experiments extracts of the nervous systems of *Balanus eburneus* were prepared using ten systems in 0.2 cc. sea water. Extracts of eyestalk of *Palaemonetes,* 24 in 0.24 cc. sea water, were also prepared to be used to produce both light-adaptational and dark-adaptational responses, known to occur (Fingerman, Lowe and Sundararaj, 1959). Injections of sea water into individuals in the third group of animals were used as controls for each experiment.

The results of these two experiments were qualitatively the same and the average distal pigment indices for each extract were averaged together. These average values for the two experiments were used to plot the curves shown in Figure 5, A. The extract of *Palaemonetes* eyestalk produced the expected light-adaptation and dark-adaptation of the distal pigment. The extract of nervous system of barnacles produced a small amount of light-adaptation and no dark-adaptation. The sea water controls remained relatively constant for the duration of the experiment.

For the second two experiments the activities of extracts of the nervous systems of *Balanus* were compared with similar extracts made from muscles of *Balanus*. Injections of sea water were used on a third group of animals as controls for each experiment. These two experiments were qualitatively the same and average distal pigment indices for each time interval were averaged together. These values were used to plot the curves shown in Figure 5, B. In these experiments all animals became more dark-adapted as the experiment progressed. This was probably due to the fact that the laboratory became darker as the experiment continued into the later afternoon. The extract of the nervous system of barnacles again produced a small but noticeable light-adaptational response, but no dark-adaptation. The extract of muscle of barnacles produced no change in the distal pigment index.

Discussion

Until recently all of our knowledge of crustacean endocrinology has been gained from studies of major groups of the Subclass Malacostraca. Carlisle and Knowles (1959) have stated that the lower groups of Crustacea have many functions "suggestive of endocrine control." The technical problems of studying these groups of relatively small animals are, of course, serious. The contribution of the present study is the demonstration that representatives of one of these lower groups, the Subclass Cirripedia, have substances in their nervous systems homologous in action to materials in the decapods when tested on certain decapods.

The most obvious property of the extracts of the central nervous system of the barnacles was found to be that of dispersing the black pigment of eyestalkless *Uca pugilator*. In three experiments described in this paper and numerous others performed in connection with other studies, the most concentrated extracts of *Balanus eburneus* that have been used have produced black-pigment-dispersal which lasts for approximately four hours.

This same property has been found in two other barnacles, *Chelonibia patula* (two experiments) and *Lepas* sp. (one experiment). Although there is a limited amount of data it appears that these second two genera contain this material in an amount 1/3 to 1/9 that found in *Balanus eburneus*. By using nervous systems from a group of barnacles for each extract the attempt was made to eliminate the influence of individual variation among barnacles. Care was also taken to insure that the assay animals in all experiments were essentially similar physiologically. It remains a possibility, therefore, that *Balanus* and *Chelonibia,* within the same family but different subfamilies of barnacles, and *Lepas,* in a separate suborder, have characteristics as different as are found in the pigmentary physiology of *Crangon* and *Palaemonetes,* which are in different families of the Suborder Natantia.

In the dilution experiment it was shown that the black-dispersing substance of *Balanus eburneus* is active in a dilution of 1/243. The activity obtained at this concentration is comparable to that obtained with the same concentration of an extract of supraesophageal ganglia of *Uca pugilator* (Sandeen, 1950).

Even though it is believed that these materials are secreted by specialized cells which are not necessarily evenly distributed throughout the nervous system, it was thought desirable to attempt a comparison of the sizes of these tissues. To do this a Whipple disc was placed in the ocular of a dissecting microscope. Using a magnification of approximately 27 the whole nervous system of *Balanus eburneus* was drawn to scale on graph paper. Using the same scale, drawings were made of the supraesophageal ganglia and circumesophageal connectives of *Palaemonetes vulgaris* and *Uca pugilator*. These nervous systems were dissected from animals of the average size used in all of the experiments. This comparison, of course, is only partially satisfactory because it is not a measurement of volume. These three drawings are shown in Figure 6.

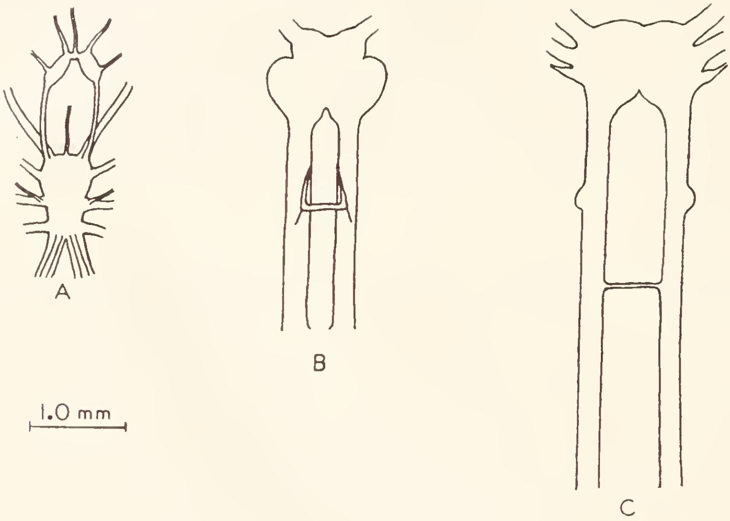

FIGURE 6. Diagrams of the central nervous system of *Balanus eburneus* (A), supraesophageal ganglia and circumesophageal connectives of *Palaemonetes vulgaris* (B), and the supraesophageal ganglia and circumesophageal connectives of *Uca pugilator* (C).

By examination of this figure it is apparent that the total area of the nervous system of *Balanus* is approximately three-fourths that of the supraesophageal ganglia of *Uca*. These tissues of somewhat comparable size give essentially similar black-dispersing activity. Until histological studies are undertaken, nothing can be said about the presence and comparative distribution of cells responsible for producing the active materials. This finding does suggest, however, that the tissue as dissected probably does not include any sort of storage organ equivalent to the sinus gland of the decapod, since an extract of the sinus gland will produce a significant black-pigment-dispersion at a dilution of 1/729 (Sandeen, 1950). The fact that the extract of *Balanus* produces a chromatophore response at a dilution of 1/243, as well as the facts that this activity is not destroyed by boiling but is destroyed by trypsin, lends support to the conclusion that we are dealing with substances homologous to those found in the decapods.

In the experiment using *Palaemonetes* as an assay animal it was shown that extracts of the nervous system of *Balanus eburneus* produce a red-pigment-dispersion and no red-pigment-concentration. When these results are compared with those obtained on the relative activities of the parts of the nervous system of *Palaemonetes* (Brown, Webb and Sandeen, 1952), some interesting contrasts are apparent. In these latter experiments extracts of all parts of the nervous system, except the circumesophageal connectives, produced greater red-pigment-dispersion (chromatophore stage of approximately 2 at 30 minutes) than did the barnacle extract (1.7 at 30 minutes). Extracts of the connectives of *Palaemonetes,* on the other hand, produced a red-pigment-dispersion, similar to that of the barnacle extract, of only about 1.7. In *Palaemonetes,* it is the circumesophageal connectives which also contain the largest amount, outside of the eyestalk, of red-pigment-concentrating activity. It was a surprise, therefore, to find low dispersing activity and yet no red-concentrating activity.

For the experiments of Brown, Webb and Sandeen (1952) the extracts contained the equivalent of one-half a portion (i.e., a supraesophageal ganglion or one connective per dose of 0.025 cc.). In these experiments on barnacles the extracts contained the equivalent of a whole nervous system in one dose of 0.02 cc. In Figure 6 it can be seen that the supraesophageal ganglia of *Palaemonetes,* as dissected for these studies, are almost equivalent in size to those of *Uca.* Since a concentration of one was used in this study, as compared to the concentration of 1/2 used by Brown, Webb and Sandeen (1952), it seems clear that the amount of red-pigment-dispersing substance present is small.

One other situation exists in which Uca black-dispersing substance occurs without the co-existence of *Palaemonetes* red-concentrating material. This is the case with extracts of the nervous system of *Limulus* (Brown and Cunningham, 1941; Snyder-Cooper, 1938). These findings lend support to the view that each of these chromatophorotropins has some kind of individuality.

Finally, it has been shown that the barnacle nervous system contains some *Palaemonetes* distal retinal pigment-light-adapting substance and no dark-adapting material. This result can be compared with that obtained by Fingerman, Lowe and Sundararaj (1959). Whereas they obtained both light- and dark-adaptation of *Palaemonetes* following injection of extracts of eyestalks of *Palaemonetes,* they obtained only a light-adapting response following injection of extract of the supraesophageal ganglia of *Palaemonetes* in a concentration of 1/3.

Barnacles are very different morphologically from the decapod crustaceans. They do not have compound eyes, nor do they have chromatophores. Pigments do occur in the mantle tissue which secretes the shell. It is possible, however, that the physiologically active substances demonstrated here do not function for the barnacle as they do for the decapod. They may serve in molting or reproduction, two functions which are hormonally regulated in the decapods. An equally likely hypothesis is that these substances which are active on the decapods co-exist with related compounds of barnacles having their own functions, and thus simply describe another systematic affinity among crustaceans. The very interesting finding of Fingerman and Mobberly (1960) that blind cave crayfish with no retinal pigments and no chromatophores have both distal-pigment-light-adapting hormone and red-pigment-concentrating material causes one to consider a third possibility, that these materials in barnacles are vestiges from a primitive ancestor that employed them in a way similar to that of modern decapods.

Summary

1. A *Uca* black-pigment-dispersing substance has been extracted from the nervous systems of three genera of barnacles, *Balanus eburneus, Chelonibia patula* and *Lepas* sp. This substance is heat-stable and can be inactivated by trypsin.

2. The *Uca* black-pigment-dispersing substance from *Balanus eburneus* is active in a dilution of 1/243. This activity is comparable to that known to be produced by a similar extract of supraesophageal ganglia of *Uca pugilator*.

3. The nervous system extracts of these barnacles do not contain *Palaemonetes* red-pigment-concentrating material, but do contain a small amount of *Palaemonetes* red-pigment-dispersing material.

4. *Palaemonetes* distal retinal pigment-light-adapting principle was demonstrated to be present in small quantity but distal retinal pigment-dark-adapting principle was not shown to be present.

5. The relationship of these findings to some of our knowledge of crustacean hormones is discussed.

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A CYTOLOGICAL STUDY OF THE ORIGIN OF MELANOPHORES IN THE TELEOSTS[1]

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Conclusive evidence for the neural crest origin of all teleost melanophores does not exist despite the growing body of evidence that the neural crest does give rise to melanophores in many fish species. Extirpation, explantation, and transplantation experiments by a number of investigators on a variety of species definitely indicate that the neural region of teleost embryos is a source of future melanophores (Lopashov, 1944; Humm and Young, 1956; Kajishima, 1958). Descriptive findings, from one of the first suggestions that melanophores stem from neural ectoderm (Borcéa, 1909), to the recent extensive work of Orton (1953), lend further support to the concept that the neural crest is a source of melanophores in fish. In fact, a tabulation of the data from the literature cited in the present paper shows some sixty species of fish, representing more than twenty families and ten orders, in which there is some evidence for the neural crest origin of melanophores; however, none of this work constitutes evidence that the crest is the sole source of these cells. Conversely, Trinkaus (1951) has removed the entire embryo at very early stages, and the remainder of the egg produced a normal complement of melanophores simultaneously with their appearance in unoperated eggs. The species used in his study was *Fundulus heteroclitus,* of which Stockard (1915) has given a detailed description of the origin of melanophores from the extra-embryonic germ ring. Stockard's observations have been supported by other authors (Gilson, 1926; Russel, 1939). Further evidence that the germ ring may give rise to melanophores is contributed by the experimental studies of Oppenheimer (see 1949), although she does not agree that this structure is a normal source of melanophores. A search of the cited literature yields evidence for this secondary site of pigment cell origin in only *F. heteroclitus* and possibly *Epiplatys fasciolatus* (Oppenheimer, 1938). Even *Fundulus majalis* seems to lack these cells, judging from the description in Bancroft (1912). These exceptional pigment cells are found largely if not solely in the extra-embryonic yolk sac membranes of a very few species of cyprinodont fishes, and in *F. heteroclitus* they all eventually encircle the blood vessels of the yolk sac—a phenomenon, which is unusual and possibly unique among fish embryos (Stockard, 1915).

There are still numerous problems concerning the origin of fish melanophores, however. Fish do not have a morphological neural crest as seen in the higher vertebrates. The experiments cited above involved removal of pieces of the ectoderm plus some of the underlying neural keel, and hence are not truly analogous with removal of the neural folds in higher forms. The descriptive data are confined to reports of the melanophores or their immediate precursors first being seen in the "neural region." Newth (1951, 1956) has studied the crest and its derivatives in the lamprey. He describes the crest as a histologically distinct region between the keel and the overlying ectoderm. Kajishima (1958) mentions very briefly that he sees a similar region in the goldfish, but he does not figure it. There appears to be no published study identifying the neural crest of teleosts and determining its extent and time of migration. Goodrich (1950) is even led to state (p. 18) that "there does not at present appear to be available critical evidence to demonstrate the origin of fish chromatophores from the neural crest. . . ."

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[1] This investigation was carried out during the tenure of a Predoctoral Fellowship from the National Cancer Institute, United States Public Health Service.

Concerning the extra-embryonic origin referred to above there is even less unanimity. Many of the descriptions predate DuShane's (1935) demonstration of the neural crest origin of amphibian pigment cells. Oppenheimer and Trinkaus have worked on this problem more recently, and Trinkaus (1951) contends there is a normal extra-embryonic source of pigment cells in *Fundulus heteroclitus*, while Oppenheimer (1949), working on the same species, attributes her results to a "bedeutungsfremde Selbstdifferenzierung" of cells in an abnormal situation.

The object of the present investigation is to describe the neural crest of several species of teleosts and to provide some information on its time of migration. The yolk sac melanophores of *F. heteroclitus* are identified at an early stage and followed throughout their differentiation to demonstrate more conclusively that in this species, and possibly in a few others, there is normally an extra-embryonic source of melanophores.

MATERIALS AND METHODS

The embryos of several species of fish were used for this study. Much of the work on the neural crest was done on the zebra fish (*Brachydanio rerio*), a tropical cyprinid which is easily raised and bred in the laboratory. Its eggs are small (0.6 mm. in diameter) and transparent enough for observation with the phase microscope. The egg is reasonably good for histological work, too, since the chorion is easier to remove than those of most fishes, and it offers less difficulty in sectioning than the larger eggs.

The eggs of *Fundulus heteroclitus*, a cyprinodont, and those of the cunner (*Tautogolabrus adspersus*), a labrid, were studied at the Marine Biological Laboratory in Woods Hole, Massachusetts. *Fundulus* has an egg which is approximately 2 mm. in diameter, large enough for some experimental work, but sufficiently transparent to allow good observation of the cells on the yolk sac in the living egg. The cunner has a smaller egg, less than 0.5 mm. in diameter, and it has been described as "glass clear." It is pelagic and too delicate for most kinds of work.

Several other tropical fish were used briefly. Their taxonomy and handling were based mostly on Axelrod and Schultz (1955). The methods used with *Fundulus* and the cunners were taken from Costello *et al.* (1957). The histological procedures in the latter reference (page 225) were used with many variations. Flemming's seems to be a more satisfactory fixative, and an isopropyl dehydration and clearing can be substituted for the ethanol-amyl acetate procedure. The stain most frequently used was Ehrlich's hematoxylin and eosin. When it was desirable to see the earliest pigment granules, safranin in 50% ethanol was used to allow visualization of the nuclei and cell outline. Basophilia was judged by eye on sections stained in pH 4 azure-B. Sections were cut at 6 μ, or thinner when better resolution was desired. Embryos to be examined whole were fixed in Stockard's (1915) fixative.

Since the pigment and propigment cells form a rather diffuse tissue, if they may be called a tissue at all, sectioned material is not ideal for observing them. Any plane of sectioning through the embryo would give good results for only a few of these flattened and irregularly shaped cells. A procedure was developed which allowed satisfactory observation of these cells in their entirety on the *Fundulus* yolk sac. The live embryo was laid on its side on a clean coverslip. The yolk sac was punctured on its top surface, and the yolk and fluid around the embryo drawn away with filter paper leaving the embryo flattened out on the coverslip. The coverslip with the embryo on top was set on an aluminum rod, the other end of which was immersed in liquid nitrogen (dry ice-acetone mixture when liquid nitrogen was not available). The embryo was thus frozen almost instantaneously. Under a dissecting microscope the top layers of the frozen embryo were carefully shaved away with a sharp scalpel or razor blade. Ideally, a layer consisting of most of one side of the embryo but only a few cell layers thick could be left on the coverslip. This tissue was then instantaneously thawed and fixed by dropping the coverslip into acetic-alcohol (1:3). The fixative precipitates the protein so that the tissue adheres to the glass. It is subsequently treated as sectioned material.

TABLE I

Zebra fish stage sequence at 28° C.

Stage No.	Age in hours	Distinguishing characteristics (pigment cell appearance)	Hisaoka and Battle stage
15	15	Blastopore closure	17
16	16+	Optic vesicles visible	17
17	18	Formation of first somites	17
18	21	Auditory placodes formed, ten somites	18
19	23	Cavity in central nervous system	18
20	27	Partial constriction of yolk mass (First pigment granules appear)	19
21	30	Motility of embryo (Visibly pigmented eye)	20
22	33	Beginning of heart beat (First body melanophores visible)	21
23	35	Circulation begins (Melanophores appear on yolk)	22
24	39	Circulation on yolk sac (Great increase in general pigment)	22
25	45	Prominent fin buds (Some expanded melanophores on body and yolk sac)	22

Living embryos were observed and staged with a dissecting microscope having a highest magnification of 80 ×. A phase contrast microscope was used extensively. It was found that the Zeiss 40 × oil phase objective made it possible to observe many intracellular details in the living fish embryos. A filar ocular micrometer was used to obtain measurements on fixed material.

At the time this study was begun, there was no set of normal developmental stages for the zebra fish and cunner. Oppenheimer's (1937) stages were used for *Fundulus*. Since there is no stage sequence available for the cunner, the observations presented here are based on the age of the embryos in hours at approximately 17° C. A modification of Oppenheimer's stages was developed for the zebra fish; however, they cover only the period extending from the closure of the blastopore to the complete differentiation of the earlier pigment cells, and are based as much as possible on the pigment cells themselves. Recently the development of the zebra fish has been described (Hisaoka and Battle, 1958), but the stages given are too coarse for the purposes of the present study. Table I presents the stages used herein and a comparison of them with those of Hisaoka and Battle.

OBSERVATIONS

The teleost neural crest

In the zebra fish, the melanoblasts begin to migrate before they become pigmented. The first melanin that can be seen is in the eye, and it is followed by a wave of melanogenesis progressing posteriorly. Since there are slight differences in the time of appearance of melanin and since the crest also shows some modification in the head region and in the tail bud, the sequence of events to be described will be concerned mainly with the trunk region.

At stage 15, the embryonic axis of the zebra fish is represented only by a swelling in the midline of the embryonic shield with some separation of the notochord (Fig. 1). As stage 16 arrives, there is a well developed neural keel in the brain region, while in the trunk the keel is represented by a ridge in the ectoderm above a well delimited notochord (Fig. 2). Figures 3 to 8 show the trunk region in stages 17 to 24. It will be seen that the neural primordium becomes progressively larger and more discrete. Initially the dorsal surface of the keel is broadly attached to the ectoderm (Figs. 3 and 4). This attachment narrows in stage 18 (Fig. 5), and the cells constituting it become loose in stage 18 to 19 (Fig. 6). It is these cells that apparently represent the neural crest of the zebra fish embryo. In Figure 7, early migrating cells can be seen leaving the crest, and the first pigment granules are forming in some of these cells. Figure 8 shows the paths of migration outlined by pigmented cells, most of which have now ceased migration.

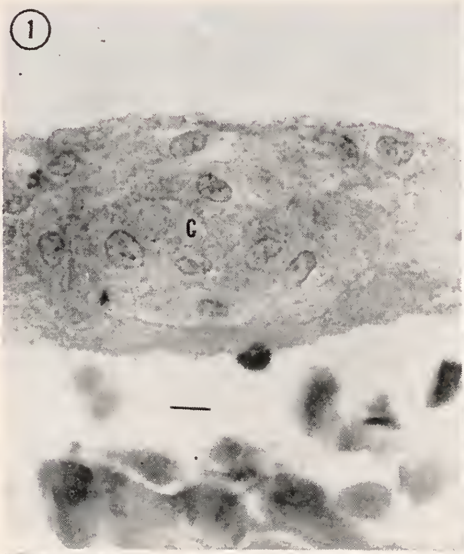
The majority of the melanoblasts first migrate between the neural tube and the somites and then down onto the surface of the notochord. From there some of them follow the remnants of what has been referred to as the segmentation cavity, to the yolk sac. Most of the cells that have become heavily pigmented along this route cease their migration and are eventually associated with various internal organs. Another path of migration is between the ectoderm and the somites. The cells in this position are easily visible with the phase microscope (Figs. 9 and 10), and are the ones that form the larval pigment. The paths of migration are quite similar to those described for the higher vertebrates.

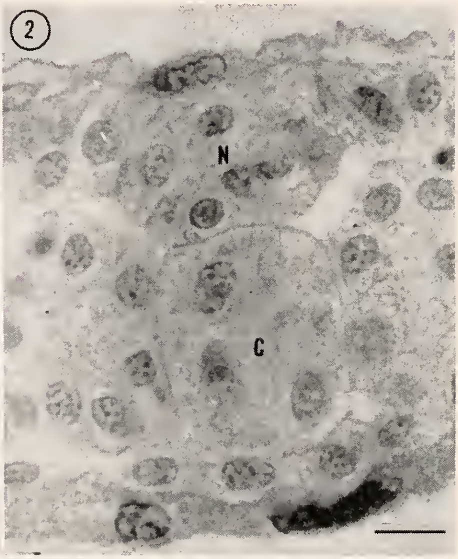
Cytologically these cells are not as desirable for the study of melanoblast differentiation as those of the *Fundulus* yolk sac which will be described in a later section; however, some of the same phenomena are observable. Prior to

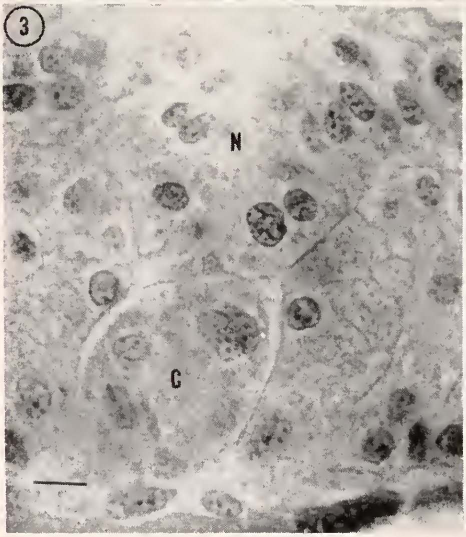
Symbols Used in Figure Legends

B—Melanoblast
C—Notochord
E—Endothelial cell
N—Neural primordium
M—Melanophore
X—Neural crest

The scale marker is 10 μ long in all figures except Figures 17 and 23 where it is 100 μ.







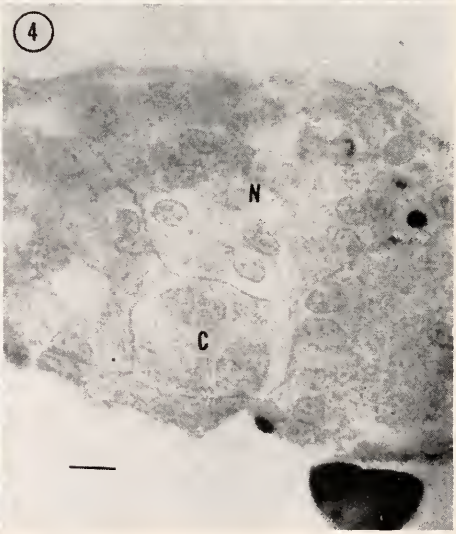
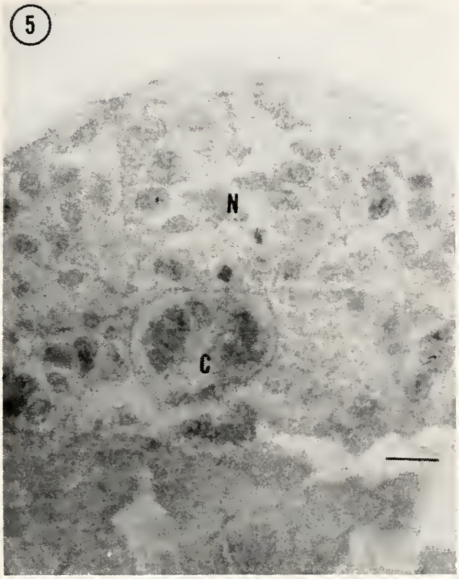


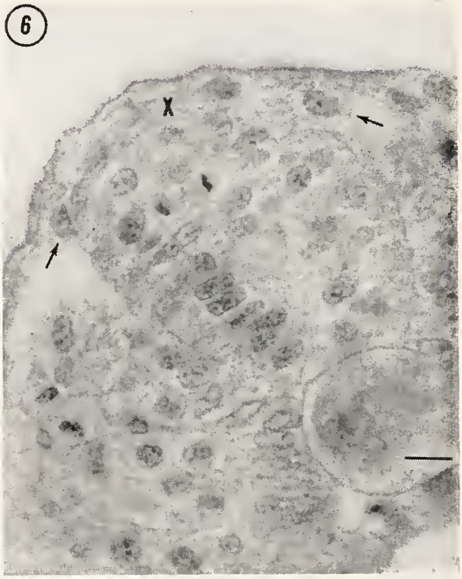
Figure 1. Zebra fish: cross-section at stage 15 showing the thickening in the midline of the embryo, which is the beginning of the neural primordium.

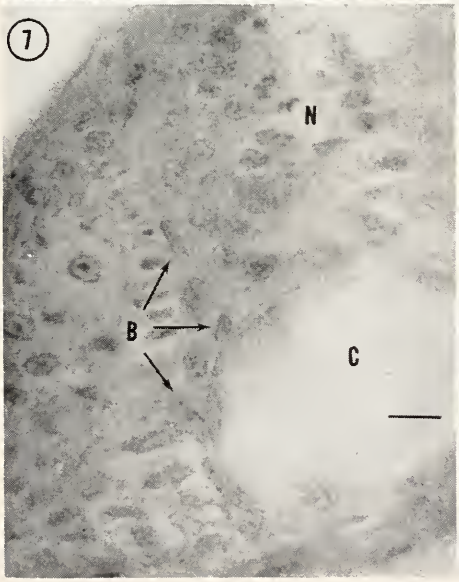
Figure 2. Zebra fish: cross-section at stage 16. The notochord is now distinct and the neural keel is beginning to form.

Figure 3. Zebra fish: cross-section at stage 17. The neural keel is enlarging.

Figure 4. Zebra fish cross-section at stage 17+. The keel is here quite large, but still broadly attached to the ectoderm.







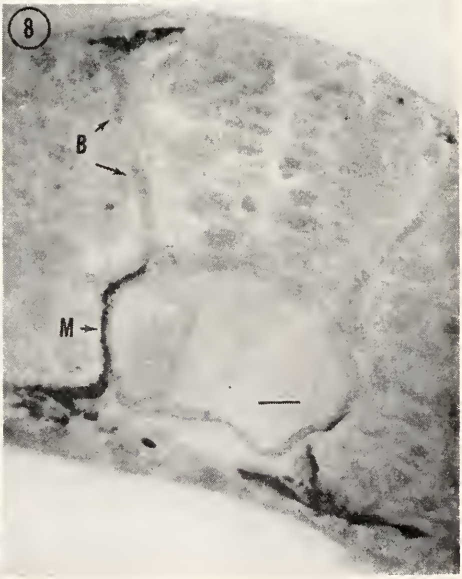
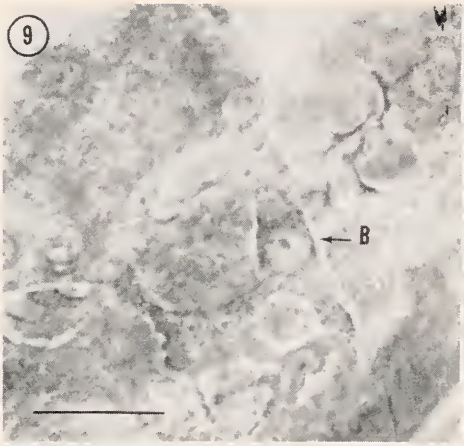


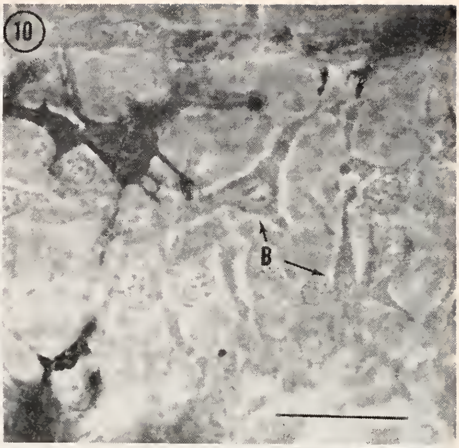
FIGURE 5. Zebra fish: cross-section at stage 18. The attachment of the keel to the ectoderm is becoming narrower.

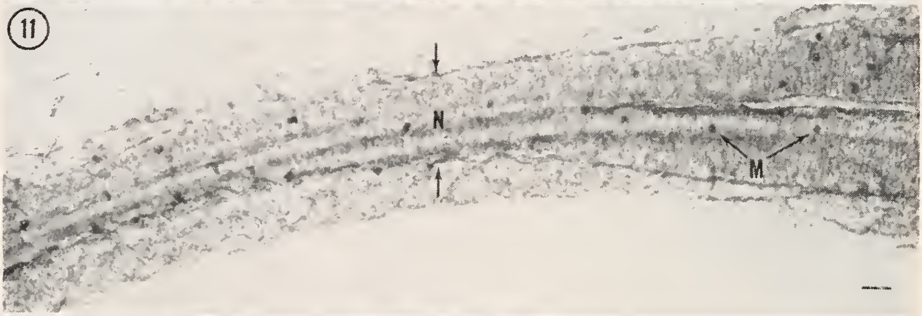
FIGURE 6. Zebra fish: cross-section at stage 18+. This is the loose crest stage. The cells indicated (arrows) will migrate shortly.

FIGURE 7. Zebra fish: cross-section at stage 19. Some of the migrating cells (arrows) contain the first melanin granules.

FIGURE 8. Zebra fish: cross-section at stage 24. The main paths of pigment migration are outlined by melanophores.







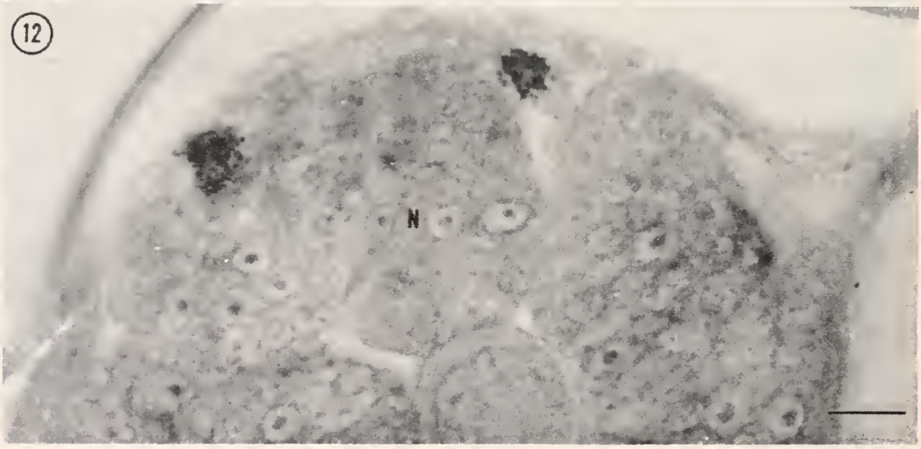


FIGURE 9. Zebra fish: phase photomicrograph at stage 24. Note the large nucleus and nucleolus of this melanoblast.

FIGURE 10. Zebra fish: phase photomicrograph at stage 24. This and the previous figure show cells in the intact, living embryo.

FIGURE 11. Dorsal aspect of a whole mount of a 30-hour cunner embryo. The width of the neural keel is indicated. The melanophores are confined to the area between the ectoderm and the keel.

FIGURE 12. Cunner: cross-section of a 20-hour embryo. Except for the presence of melanophores here, this closely resembles the loose crest stage of the zebra fish (Fig. 6).

melanogenesis there is a great increase in cytoplasmic basophilia. The nuclei triple or quadruple their volume during differentiation and contain large, densely basophilic nucleoli (Fig. 9). The cell size also increases enormously, although the very irregular shape (Fig. 10) makes calculations difficult. In general, the differentiation of the neural crest pigment cells in the zebra fish resembles that of the *Fundulus* yolk sac pigment which is not of neural crest derivation.

The pigment of the pelagic cunner egg is interesting because in similar embryos Orton (1953) and others have observed melanophores forming in the crest region well before any pigment cell migration. Figure 11 shows a 30-hour cunner embryo. The dorsal pigment has been visible for a number of hours, but the cells have not migrated. There is no pigment elsewhere in the embryo. The melanophores are still in close association with the neural tube which is beginning to form its cavity in the hind-brain region. A cross-section through an earlier cunner embryo (Fig. 12) shows the relationship of the melanophores to the neural keel. This stage resembles the loose crest stage of the zebra fish embryo.

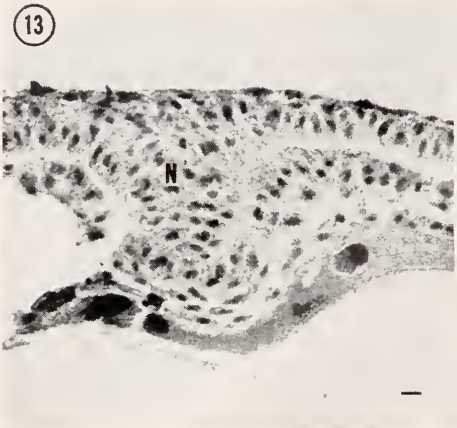
The neural crest cells of *Fundulus* begin to migrate before melanogenesis occurs. Figures 13 to 16 show several stages in the development of the neural region of this form. By stage 14 the beginnings of the neural primordium are present. During stages 15 to 17 the keel develops rapidly (Figs. 13 and 14), and by late stage 16 or early 17 the crest becomes fairly distinct histologically. This stage may correspond with the loose crest stage of the zebra fish (Fig. 14). Between stages 17 or 18 and stage 21 crest migration begins in the trunk region (Fig. 15). Some crest migration may have started a little earlier in the head region, but it is difficult to distinguish head mesenchyme from crest cells. Sometimes at stage 18 early melanophores are visible in the head mesenchyme, possibly associated with the eye, but there are none in the trunk. Migration continues and melanization begins in the trunk during stages 19 and 20 (Fig. 16). In the discussion which follows, migrating cells are described on the yolk prior to stage 15. Some of these cells may be identified as melanoblasts at stage 16, and melanogenesis has begun in them by stage 18. Not until several hours later is the first melanin seen in the embryo proper. Thus, the yolk sac melanophores apparently are not derivatives of the neural crest.

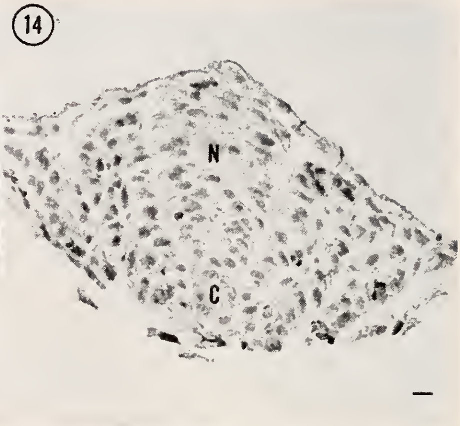
The yolk sac melanophores

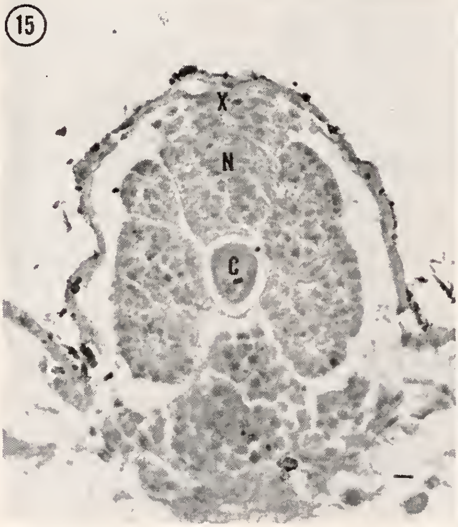
On the yolk sac of *Fundulus heteroclitus,* Stockard (1915) described wandering mesenchymal cells which eventually developed into four different cell types: black and brown pigment cells, red blood cells, and endothelial cells. These cells are readily observed with phase optics, and additional detail can be seen using the freezing-shaving technique described in this paper. The following description is based on these preparations.

The cells described by Stockard begin to migrate shortly before the closure of the blastopore (Oppenheimer stages 14 to 15) from the germ ring and possibly from the caudal mass and sides of the embryo. At these stages the cells are quite similar, and it is difficult to determine which types come from a given source. The migration continues until after the blastopore closes, at which time cells may be seen leaving the remnants of the germ ring (Fig. 17).

Stockard was not certain that his identification of these cells on the basis of







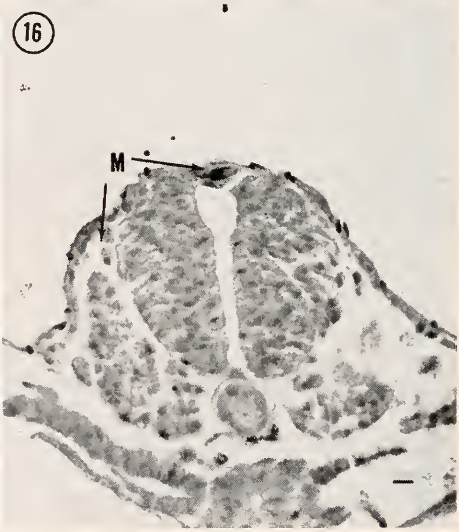


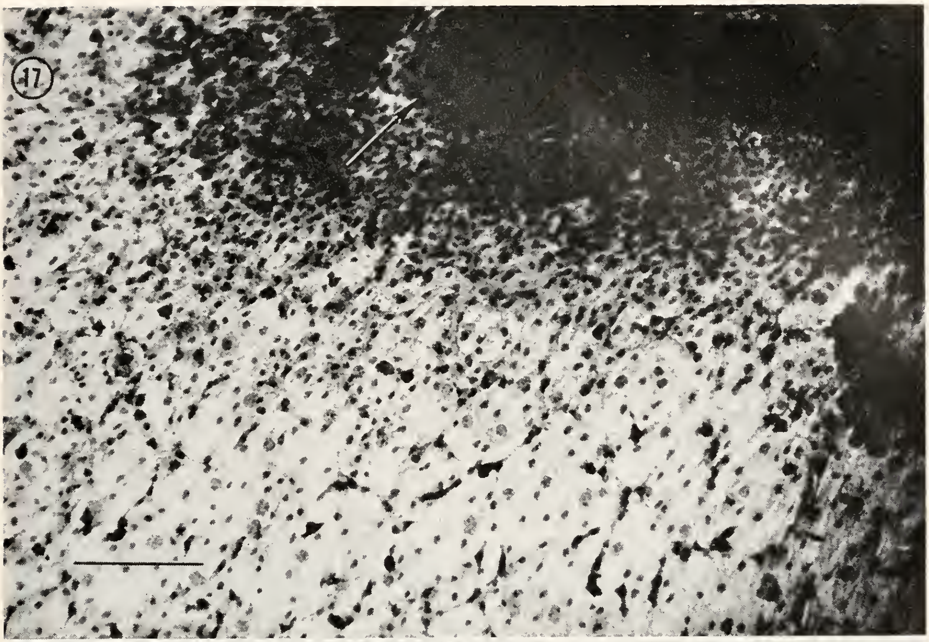
FIGURE 13. *Fundulus:* cross-section at stage 15. The neural keel is forming.

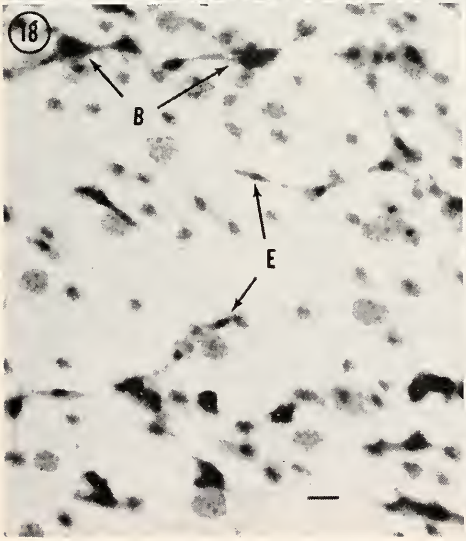
FIGURE 14. *Fundulus:* cross-section at stage 18. There is a well developed neural keel with loosening of the crest cells.

FIGURE 15. *Fundulus:* cross-section at stage 21. This is a late loose crest stage in the posterior trunk region.

FIGURE 16. *Fundulus:* cross-section at stage 21. The crest is migrating and melanophores are beginning to differentiate in this embryo. There are well developed yolk sac melanophores before this stage.

size and shape was fully warranted, and he suggested the possibility that some of the cells might be able to change from one type to another before their final differentiation. In the present study, however, it has been found that these wandering cells can be distinguished from each other on the basis of several cytological properties, and the changes in these characteristics were followed through the differentiation of the cells. (The brown pigment cells were not carefully





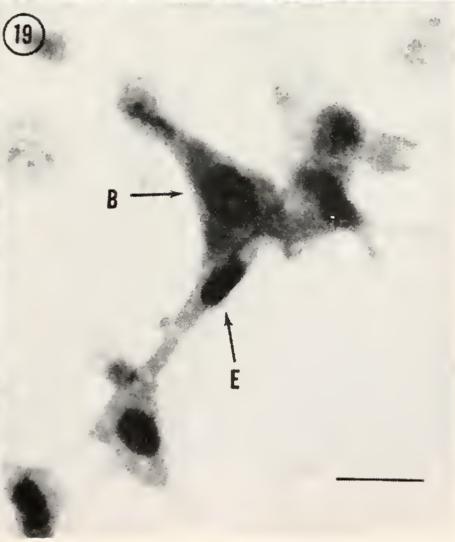
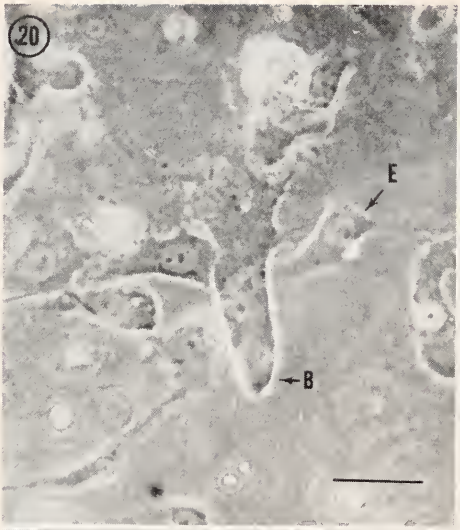
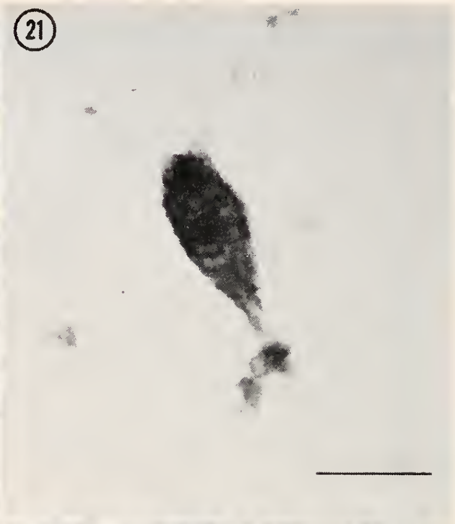


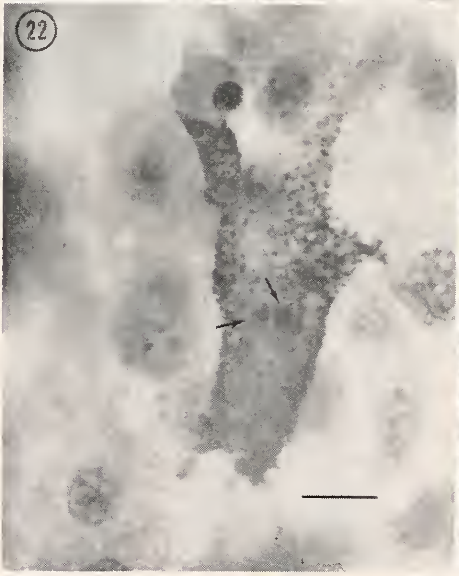
FIGURE 17. *Fundulus:* frozen-shaved preparation at stage 16. The caudal end of the embryo is at the arrow. The darkly stained cells are the wandering cells of Stockard while the lightly stained nuclei belong to the ectoderm. The scale line equals 100 μ.

FIGURE 18. *Fundulus:* frozen-shaved preparation at stage 16. The endothelial cells may be distinguished from the melanoblasts.

FIGURE 19. *Fundulus:* frozen-shaved preparation at stage 16. The large nucleus and nucleoli and the basophilic cytoplasm of the melanoblast are apparent here.







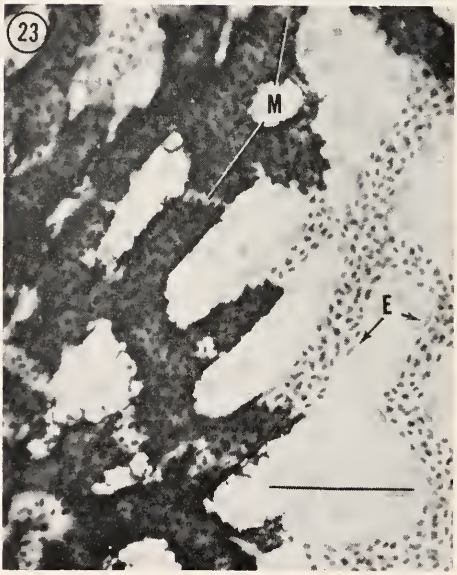


Figure 20. *Fundulus:* Phase photomicrograph of living stage 17 embryo. Note the cytoplasmic granularity of the melanoblast.

Figure 21. *Fundulus:* frozen-shaved preparation at stage 17+. Note the intense basophilia of this azure-B-stained melanoblast.

Figure 22. *Fundulus:* frozen-shaved preparation at stage 20. The nucleoli are still large in this early melanophore (arrows).

Figure 23. *Fundulus:* frozen-shaved preparation at stage 32. The extent of one melanophore is indicated. At stage 16 these cells were the same size as the blood and endothelial cells of the blood vessels which they now encompass. The scale mark is 100 μ.

followed. They seemed to arrive later and are possibly of neural crest origin.)

The migrating cells initially are spindle-shaped with lightly staining cytoplasm. Their nuclei are spherical, approximately 5 μ in diameter (70 μ^3 in volume), containing several small nucleoli. The endothelium and blood cells subsequently "lose" their nucleoli at stages 17 and 19, respectively. The blood cells become small and spherical with sharp, radiating pseudopods. They have a granular cytoplasm by stage 20, and there is little change in their nuclear volume. The endothelial cells retain their spindle shape, but stain less intensely than the original cells. Even after these cells expand to form the blood vessels of the yolk, their nuclei show little increase in volume.

The changes in the melanoblasts are quite striking, however. Figures 18 to 23 show stages in the differentiation of these cells. The most dramatic early changes are in the nuclei. The nucleoli increase in size and basophilia until the onset of melanogenesis (Figs. 19 to 22). At this time the nuclear volume is approximately 250 μ^3, and it increases further until in the fully differentiated melanophore it is almost 600 μ^3. This represents a nuclear volume increase of almost ten times over that of the initial cells. The cytoplasm of these cells becomes quite basophilic as compared with the other wandering cells (Fig. 21). A granularity also appears at stage 17 (Fig. 20), possibly representing propigment granules. The over-all cell size increases rapidly during differentiation until the final immense size of the expanded melanophore is reached (Fig. 23). These cells may thus be identified and followed with a fair degree of certainty from stage 16 through their final differentiation, and at stage 16 there is no sign of neural crest migration except rarely in the head region.

A brief search was made in the tropical fish stores and the streams of the Chicago area for other cyprinodont species which appeared to have the "non-neural crest" pigment. Simple observational criteria were used. (1) Are there numbers of relatively large wandering cells on the yolk sac before pigmentation begins? (2) Do melanophores appear evenly distributed over the yolk sac before the body pigment appears? (3) Do the yolk melanophores eventually wrap around the blood vessels of the yolk sac? The only species found which was affirmative for all three criteria was *Fundulus dispar*. *Epiplatys chaperi* was affirmative for the first two and was the only other species in which there appeared to be a secondary site of melanophore origin. Two species of *Amphyosemion* appeared to have neural crest pigment which migrated onto the yolk sac before melanogenesis began. Several other cyprinodonts had little or no yolk pigment, and no representative of the five other orders briefly studied satisfied any of the criteria. This sample, however, may be too small to make valid generalizations about the teleosts as a whole.

Discussion

In the higher classes of vertebrates where the neural tube is formed by neural folds, the neural crest might be viewed as the ectodermal tissue connecting the neural primordium with the general ectoderm. Although the neural keel of the teleosts is formed in a different manner, the crest occupies a position between it and the ectoderm. As the crest migrates, the dorsal border of the neural primordium becomes progressively more distinct until finally all attachment to the ectoderm is lost.

Since several investigators have demonstrated that the neural crest is the sole source of melanophores in most of the higher vertebrate classes (see DuShane, 1946), it is frequently stated that all vertebrate pigment cells have the same origin (see Rawles, 1948, p. 405). There is good evidence for the neural crest origin of pigment in the lampreys (Agnatha) (Newth, 1956) and fair evidence for the teleosts in general.. As yet, however, there appears to be no evidence with regard to the Chondrichthyes or the Reptilia.

The relatively minor exception to the above generalization provided by *Fundulus* does, however, make a more thorough experimental study of the teleosts seem desirable. For lack of confirmation or completeness, none of the work cited constitutes adequate proof that the neural crest is the sole source of melanophores in any species of fish, while there appears to be good evidence in one species that some melanophores have a different origin.

The significance of the exception cannot be easily evaluated at present for several reasons. First, it is not known how frequently it occurs in other forms, although it does appear to be relatively rare. Secondly, the source of these exceptional melanophores has not been conclusively demonstrated, although any direct relationship with the neural crest is unlikely. Thirdly, the adaptive significance of the yolk sac pigment is unclear.

The first point above has been discussed elsewhere in this paper, and there is nothing more to add here. With regard to adaptive significance, if some fish species require either earlier pigment or pigment of different characteristics than that provided by the crest (see Rass, 1937), then the occurrence of such pigment may be of little general significance. If, instead, relatively undifferentiated tissues such as the germ ring have the potentiality of differentiating into melanophores (see Barth *et al.,* 1960), it might be found that many unrelated species have some non-neural crest pigment cells.

A certain amount of work has been done that bears on the second point above. Stockard states that most of the "wandering mesenchyme cells" come from the germ ring and its remnants after closure of the blastopore, and a smaller number come from the lateral sides of the embryo, especially at its caudal end. The differentiation of these cells does not take place until they are widely spread over the yolk sac, and it is difficult to say where they came from. Possibly they could be followed individually or traced by vital staining. Empirically, the germ ring seems the most likely source of the melanophores. It contributes most of the cells, and although there is disagreement in the literature concerning the fate of the germ ring (Lewis, 1912; Oppenheimer, 1938; Brummett, 1954), Oppenheimer's experiments demonstrate that it has broad potentialities for differentiation under experimental conditions. Perhaps it is simplest to consider the germ ring a totipotent remnant of the blastodisc which has felt comparatively little influence from the gastrulation of the embryo proper. That the embryo has little influence is borne out by the experiments of Trinkaus (1951) and by the fact that anomalous eggs may be found occasionally in which there are normal yolk sac membranes with melanophores, but no sign of embryonic structures.

It is interesting to note that the cytological aspects of differentiation are similar in both the neural crest and non-neural crest melanophores, and that in both the signs of greatest cellular activity precede the onset of melanogenesis. Quantitative studies of cytoplasmic basophilia or nucleolar basophilia and size would be expected to follow much the same curve as the one for oxygen consumption given by Flickinger (1949)—reaching a peak at the onset of melanogenesis and falling off thereafter. Such data tend to indicate that in the melanophore, at least, the processes of differentiation may take more cellular "effort" than the elaboration of the materials characteristic of the differentiated cell.

Much of the material in this paper was submitted as a dissertation for the degree of Master of Science at the University of Chicago. The author wishes to express his thanks to his sponsor, Dr. Hewson Swift, for help on many aspects of this study.

Summary

In this study, three species of fish which differ in the developmental histories of their pigment cells have been used—the zebra fish (*Brachydanio rerio*), the cunner (*Tautogolabrus adspersus*), and *Fundulus heteroclitus*. Their melanophores have been studied with respect to their origins and subsequent histories, including some of the cytological events occurring during differentiation.

1. In the teleosts, the neural crest has been identified as the tissue connecting the neural keel with the overlying ectoderm. During development, this tissue loosens, and the cells migrate from this region. In some of these cells melanogenesis has already begun.
2. Differentiated melanophores are demonstrated in the neural crest of the cunner embryo well before migration occurs.
3. It is concluded that the normal embryonic pigment of the teleosts has its origin in the neural crest, as in other vertebrates.
4. In some fish, notably in *Fundulus*, the pigment cells of the yolk sac are not of neural crest origin. Instead they appear to arise from the extra-embryonic germ ring. These cells may be identified on the yolk sac several hours before there is histological evidence of crest migration.
5. Many of the cytological aspects of differentiation are similar in the melanophores, regardless of their site of origin.

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CERCARIA DIPTEROCERCA MILLER AND NORTHUP, 1926 AND STEPHANOSTOMUM DENTATUM (LINTON, 1900) MANTER, 1931 [1]

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After a preliminary survey had shown that *Nassa obsoleta* is the most heavily parasitized gastropod in the Woods Hole region, Miller and Northup (1926) reported on a study conducted for one year to determine seasonal infection of this snail by larval trematodes. They examined 8875 individuals, first by isolation for 48 hours to determine those from which mature cercariae were emerging, and subsequently by crushing the snails individually and examining the tissues under a binocular microscope for infection by the asexual generations. They recorded total and seasonal infection by five species, all described as new to science, and named *Cercaria setiferoides*, *Cercaria quissetensis*, *Cercaria variglandis*, *Cercaria dipterocerca*, and *Cercariaeum lintoni*. The last species had been described but not named by Linton (1915).

Later studies have traced the life-cycles and systematic relations of these cercariae. Martin (1938) showed that *C. setiferoides* encysts in turbellarians and spionid annelids and becomes mature in the sand-dab, *Hippoglossoides platessoides*, and small flounders (not specifically identified but probably *Pseudopleuronectes americanus* or *Paralichthys dentatus*). The adults were described as *Lepocreadium setiferoides* (Miller and Northup). Stunkard (1938a) worked out the life-cycle of *C. quissetensis;* the cercariae encyst in marine mollusks and become sexually mature in the herring gull, *Larus argentatus*. They were described as *Himasthla quissetensis* (Miller and Northup, 1926). Stunkard (1938b) found that *C. lintoni* had been described and named *Distomum lasium* by Leidy (1891); that the metacercariae encyst in polychaete annelids and mature in fishes. The worms were assigned to *Zoogonus rubellus* (Olsson, 1898) Odhner, 1902, but after study of the European species, whose metacercariae were found in sea-urchins, Stunkard (1941) recognized bionomic and morphological differences between the European and American species and listed the adults of *C. lintoni* as *Zoogonus lasius* (Leidy, 1891). Stunkard and Hinchliffe (1952) discovered that *C. variglandis* is the causative agent of "swimmers' itch" of oceanic beaches in New England and the adults proved to be identical with avian blood-flukes that had been described by Price (1929) as *Microbilharzia chapini*. Penner (1953) suppressed *Microbilharzia* as a synonym of *Austrobilharzia* Johnston, 1917 and listed the species as *Austrobilharzia variglandis* (Miller and Northup, 1926). The fifth species, *Cercaria*

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stomach of *Lophius piscatorius* which also contained two partially digested specimens of the dab, *Limanda ferruginea.*

Other encysted larvae from various parts of the world have been referred to *Stephanostomum* on the ground of morphological similarity but without experimental confirmation. They were found in Britain by Johnstone (1905), Lebour (1908), Nicoll and Small (1909) and Nicoll (1910); in Ceylon by Lühe (1906), in Japan by Yamaguti (1934, 1937) and in Florida by Sogandares-Bernal and Hutton (1959a). Martin (1939) reported the first experimentally demonstrated life-cycle in the genus. He showed that sticky-tailed, stylet-bearing, ocellate cercariae from rediae in *Nassa obsoleta* encyst and develop to metacercariae in the mesenteries and liver of *Menidia (Menidia) notata* and continue development to nearly mature adult worms in the intestine of young puffers, *Spheroides maculatus.* The worms were identified as *Stephanostomum tenue* (Linton, 1898) Martin, 1938, a species described originally from the striped bass, *Roccus lineatus,* at Woods Hole, Massachusetts, and subsequently (Linton, 1940) from the sand-launce (*Ammodytes americanus*), the sea-raven (*Hemitripterus americanus*), the kingfish (*Menticirrhus saxatilis*), the white perch (*Morone americana*), the striped bass (*Roccus saxatilis*), and the toadfish (*Opsanus tau*). Margarita Bravo (1956) reported *S. tenue* from the intestine of *Trachurops crumenophthalma* taken on the Pacific coast of Mexico.

The life-cycle of a second species, *Stephanostomum baccatum,* was reported by Wolfgang (1954, 1955a). According to the report, ophthalmoxiphidiocercous cercariae, "secondarily" lacking stylets, develop in rediae in *Buccinum undatum* and *Neptunea decemcostatum,* encyst as metacercariae in the skin and somatic muscles of pleuronectid fishes and occur as adults in the rectum of *Hemitripterus americanus* and other fishes. A description was given of all stages in the cycle but there are disturbing inconsistencies in the account which may invalidate the conclusions. *Stephanochasmus baccatus* (= *Stephanostomum baccatum*) was described by Nicoll (1907) on a single specimen from the intestine of *Hippoglossus hippoglossus.* The description was adequate to validate the species and was supported by a good figure. The species was reported by Manter (1926) from the same host taken at Mount Desert Island, Maine. Stafford (1904) recorded *Stephanostomum sobrinum* (Levinsen, 1881) Looss, 1901 from the sea-raven (*Hemitripterus americanus*), the arctic eelpout (*Lycodes* sp.), and the wrymouth (*Cryptoacanthodes maculatus*). Caballero (1952) intimated that the specimens are probably not identical with *S. sobrinum* and suggested that they may belong to *S. tenue.* After examination of Stafford's specimens, Wolfgang (1955a) stated that they are *S. baccatum.* Wolfgang studied approximately 1000 specimens, taken from a variety of hosts, but data from literature and from observation are intermingled and frequently it is difficult if not impossible to distinguish between them. He reported the worms from the lower intestine and rectum and listed as final hosts the three species named by Stafford and also *H. hippoglossus* and the short-horned sculpin (*Myoxocephalus scorpius*). The distribution of the parasite was given as across the North Atlantic Ocean from Maine to Nova Scotia, Newfoundland, Greenland and the North Sea coast of Europe. But Wolfgang was doubtful of the validity of *S. baccatum* since he stated (1955a, p. 119), "Levinsen inadequately described *S. sobrinum* from *Cottus scorpius* in Greenland waters, and Odhner (1905) reported but did not redescribe it from the same host in unspecified arctic waters. Although Levinsen's description of *S. sobrinum* is vague it agrees essentially with that of *S. baccatum*. In view of the hosts and the distribution of the parasite, *S. sobrinum* and *S. baccatum* are probably conspecific." Stafford may have been correct in his determination and if the two species are identical, *S. baccatum* disappears as a synonym of *S. sobrinum*. Furthermore, Wolfgang stated (1955b, p. 135), "that *S. baccatum* shows no valid differences from *S. triglae*." If these species are conspecific, *S. triglae* Lebour, 1908 also would be suppressed as a synonym.

Although Wolfgang gave descriptions of the successive stages in the life-cycle of *S. baccatum,* there is no assurance that the cycle was actually completed in experimental hosts. The methods were so lacking in control that opportunity for confusion was obvious. There is virtually no evidence that the cercariae taken by dissection from *Buccinum undatum* and *Neptunea decemcostatum* develop into the metacercariae found encysted in the flounders. Moreover, proof of the identity of the metacercariae encysted in winter flounders, *Pseudopleuronectes americanus,* and the adults of *S. baccatum* was based on infections resulting from the following procedure, (1954, p. 965), "Gelatin capsules were filled with cysts taken from fins and muscles of the winter flounder and placed in the siphons of clams which were then fed to the fish." The author admitted (p. 965), "Although several hundred cysts were used for each dose, no comparison between numbers of cysts introduced into experimental hosts and numbers of adult worms recovered could be made because their viability may have been impaired by dissection." But the cysts of *Stephanostomum* are so tough and resistant that it is difficult to see how the infectivity of the larvae could be impaired during dissection. Furthermore, it is apparent from the report that the fishes used for experiment were already infected and as many as 567 worms were taken from a sea-raven collected near Clam Cove, Deer Island, N. B. The data presented in Table I (1954, p. 966) show that after feeding several hundred cysts in each dose, the heaviest infection (123 adult worms) was found in a sea-raven dissected four days after feeding, at which time the metacercariae of experimental infection would not yet be adult, and that the number of adult worms decreased with the length of time after feeding to a single worm in an eelpout after twenty-two days, the longest time recorded. These data strongly suggest that natural infections harbored by the experimental fishes were gradually lost during captivity and there is no proof that the worms recovered at autopsy were derived from the cysts that had been fed in the experiment. But the most disturbing inconsistencies are found in the morphological section of the paper. As described, the cercariae show no significant differences from *C. neptuneae* Lebour, 1912 from the same and related hosts, and the two may be identical. An excretory duct is described as extending posteriad from the bladder and terminating in a pore at the tip of the tail. Such a condition is so unusual in a cercaria of this type that the observation is open to question. Moreover, in the cercaria the excretory collecting ducts are portrayed as mesostomate, *i.e.*, they bifurcate behind the acetabulum to give rise to anterior and posterior branches, whereas in the metacercaria they are represented as stenostomate, extending forward without branching to the level of the eye-spots. The latter condition is characteristic of species in the genus *Stephanostomum*. If the descriptions are correct, it is obvious that the cercaria and metacercaria belong to different taxonomic groups and can not be stages in the life-cycle of a single species.

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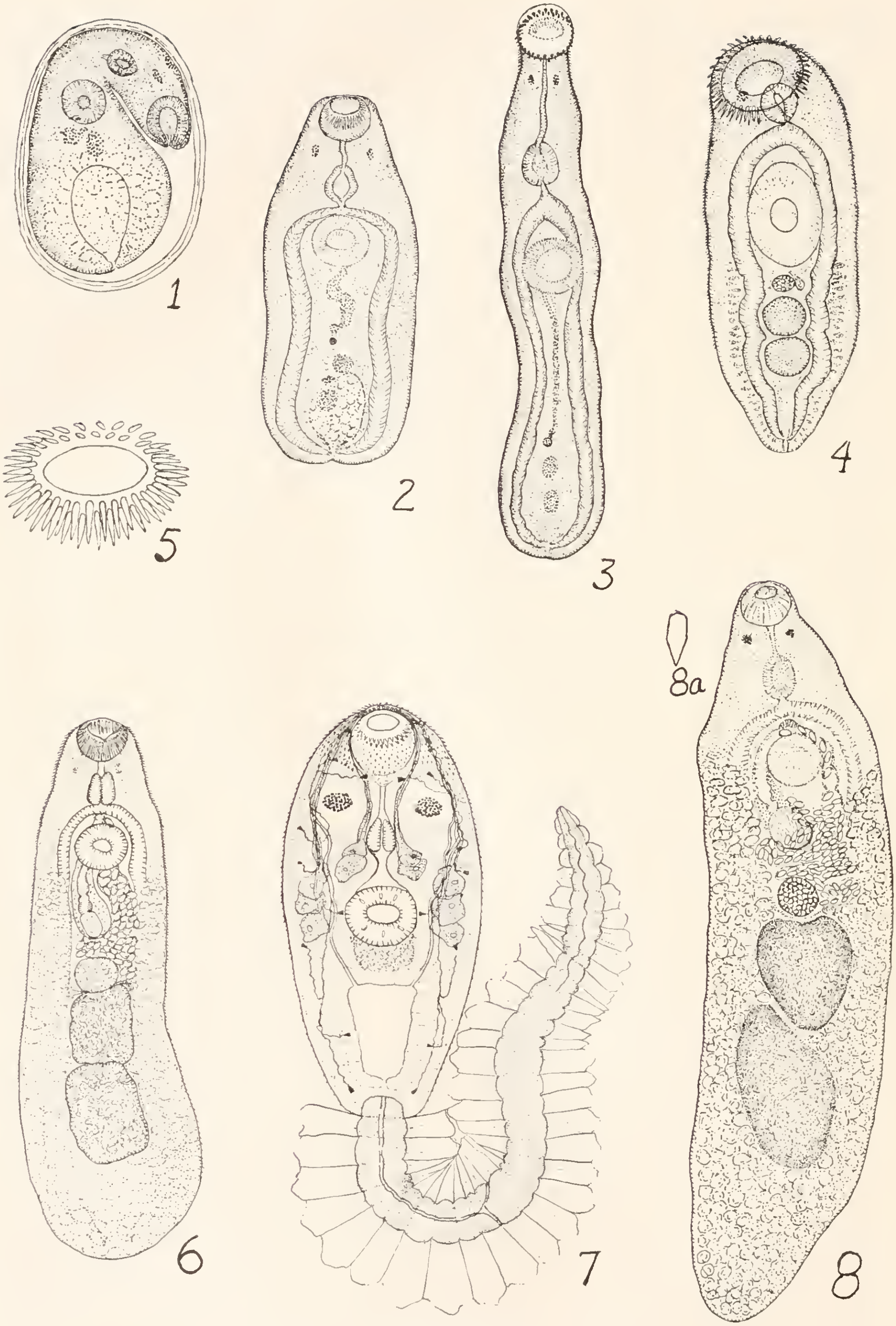
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Figures 1–8a.

Material and Methods

Nassa obsoleta, collected in the Woods Hole area, were isolated to find those which were shedding *Cercaria dipterocerca*. The incidence of infection was about 0.2% of the snails taken at the Sheep-Pen Cove on Nonamesset Island; less at other places. Miller and Northup (1926) reported a total of only four infections in the examination of 8875 snails collected at Quisset harbor. When snails are kept for long periods in fingerbowls, the number of cercariae released each day gradually diminishes, and after 12 to 16 weeks shedding may be stopped completely. But although reproduction of the parasite slows down or ceases temporarily, the infection remains in dormant condition and becomes active when the snail again has an abundance of food. It appears that a parasitized snail carries the infection indefinitely and that the parasite undergoes periods of interrupted activity during the winter or whenever the mollusk lacks sufficient nourishment to maintain both its basic metabolism and reproduction of the parasite.

Since the cercariae are provided with penetration glands, they were added to small aquaria with possible invertebrate and vertebrate hosts. They invaded small *Fundulus heteroclitus* and *Menidia menidia* but the fishes often harbored natural infections which could be confused with experimental ones. Dr. Evelyn Shaw reared *M. menidia* from eggs at the Marine Biological Laboratory, and kindly provided small fishes that had not been exposed to infection and therefore afforded absolute control of the experiments. Grateful acknowledgment is made here to Dr. Shaw. The small fishes were fed brine shrimp, *Artemia salina*. The cercariae penetrated the tissues and encysted in the throat, in the mesenteries and in the liver. They were found also under the skin and in the muscles, but the cysts were chiefly in the viscera and apparently the cercariae were taken into the mouths of the fishes with water for respiration. Dissection of these *M. menidia*

Figure 1. Metacercaria, in cyst 0.28 mm. long, from laboratory-reared *Menidia menidia*, exposed to *Cercaria dipterocerca*.

Figure 2. Metacercaria, from laboratory-reared *M. menidia* exposed to *C. dipterocerca*, released from cyst and pressed to study excretory system; worm as fixed, stained and mounted is 0.62 mm. long. This worm has 54 peristomial spines.

Figure 3. Metacercaria, identified as *Stephanostomum tenue*, released from cyst from naturally infected *Fundulus heteroclitus*, Woods Hole, Mass. Specimen fixed, stained and mounted is 1.69 mm. long. This worm has 42 peristomial spines; compared with Figure 2, note length of prepharynx and location of gonads.

Figure 4. Smallest of 12 worms on slide No. 8199, Helminthological Collection, U. S. Nat. Museum. Material of *Stephanostomum dentatum* collected 6 October 1911 by Vinal Edwards from *Paralichthys dentatus* at Woods Hole, Mass., ventral view. The worm is somewhat contracted, the anterior end curved ventrally and as mounted is 0.70 mm. long. This worm has 54 peristomial spines.

Figure 5. The peristomial spines of the largest specimen, 2.31 mm. long, on slide No. 8199, Helminth. Coll., U. S. Nat. Museum. This worm has 54 peristomial spines.

Figure 6. The largest specimen collected by the writer, *S. dentatum* from *P. dentatus*, at Woods Hole. Fixed under coverglass pressure, stained and mounted, it is 2.79 mm. long.

Figure 7. *Cercaria dipterocerca*, from *Nassa obsoleta*, ventral view, the portion of the excretory system in the tail undergoing regression.

Figure 8. Specimen, 3.5 mm. long, on slide No. 8200, Helminth. Coll., U. S. Nat. Museum. Collected 5 September 1919 from *P. dentatus*; identified as *S. dentatum* and figured in Linton (1940), Plate 3, Figure 25. Only a few peristomial spines remain; they are broad, flat, scale-like.

Figure 8a. Peristomial spine from worm shown in Figure 8.

at intervals during the ensuing six weeks yielded a series of developing metacercariae, from recently encysted larvae to mature specimens indistinguishable from those found in natural infections of *M. menidia* and *F. heteroclitus* taken in the Woods Hole area. Most of the natural infections, however, were *Stephanostomum tenue* (Fig. 3) which can be distinguished from *S. dentatum* because the circumoral spines are fewer in number and different in shape. Other differences are apparent from comparison of Figures 2 and 3.

Juvenile and adult specimens of *S. dentatum* have been found as natural infections of *Paralichthys dentatus* taken at Woods Hole. Small and medium sized representatives of this species have been kept for several weeks in the laboratory. But attempts to infect final hosts, *P. dentatus* and other flatfish, have so far not been successful, since the fishes have consistently refused to eat in the aquaria. When a larger aquarium with colder water is available, it should be possible to complete the study and obtain experimental infection of final hosts that had been held in a control tank for several weeks to insure that all worms of natural infection would be sexually mature and that those of experimental infection could be positively identified. Experimental infection of the snail host must await a supply of eggs and miracidia from infected fishes.

Study of the specimens of *Stephanostomum* on deposit in the Helminthological Collection of the U. S. National Museum was afforded through the kindness of Dr. Allen McIntosh, to whom grateful acknowledgment is made. Many of the worms were not suitable for specific identification, but slide No. 8199 contained twelve specimens of *S. dentatum*, collected October 6, 1911, by Vinal Edwards from *Paralichthys dentatus* at Woods Hole, Massachusetts. The specimens ranged from juvenile worms (Fig. 4) to fully mature, gravid individuals, and provided opportunity for comparison of these worms with metacercariae recovered from experimental hosts after exposure to *Cercaria dipterocerca*.

DESCRIPTIONS

Cercaria dipterocerca

Redia

The rediae were described briefly and figured by Miller and Northup (1926). The hemal sinuses of the snail frequently contain scores of small rediae 0.10 to 0.20 mm. in length, similar to the one shown in Figure 4 of Miller and Northup. In these small rediae the body is colorless, the pharynx 0.02 to 0.03 mm. in diameter, the digestive cecum is saccate, about one-half the length of the body, and frequently it contains orange-colored droplets. These larvae are very mobile, moving easily by alternate contractions of annular and longitudinal muscles, anchoring one region of the body while advancing another. There are no feet, specialized portions of the body-wall, which may be protruded to serve as holdfasts. Discovery of a small redia with a contained daughter-redia demonstrates more than one redial generation and the large number of small rediae suggests repeated generations of daughter-rediae.

Larger rediae, containing germ-balls and one or more cercariae, measure 0.25 to 0.75 mm. in length and 0.07 to 0.14 mm. in width. Old ones, empty except for a few germ-balls, are seen occasionally; they may measure up to 1.25

mm. in length, with long, pointed, posterior ends. The average measurement given by Miller and Northup is larger than found in the present study and may have been made on specimens under considerable coverglass pressure. In general, the rediae are oval to cylindrical, often attenuated at one or both ends. Frequently the anterior end is prolonged by a long, slender, neck-like region, about one-half the diameter of the succeeding portion of the body. Contraction of circular muscles may produce an annulate appearance or the formation of two or more wider regions separated by narrower ones. The pharynx is oval to spherical and 0.025 to 0.048 mm. in diameter; its wall is about 0.011 mm. thick. With increasing size and the maturity of increasing numbers of cercariae, the digestive ceca become relatively shorter, the body-walls become stretched and thinner, and the rediae less active. The wall contains yellow to orange droplets, one to four microns in diameter, usually associated in larger oval or circular masses, ten to fifteen microns in diameter. These masses easily dissociate. The cecum contains material, orange to greenish to blackish in color. A birth pore is present, located about the length of the pharynx behind that sucker. The cercariae move about freely in the cavity of the redia.

Cercaria (Figure 7)

Examination of unstained living specimens and others, after staining with vital dyes and after fixation, staining and mounting as permanent preparations, confirms the original description in large part and adds details of the excretory system. The two pairs of glands which stain with neutral red, situated lateral and posterior to the pharynx, were observed. There are apparently three, rather than four, penetration glands situated at either side of the acetabulum. The cells are lobed and partially overlap one another, which renders delineation difficult. Only three ducts were identified although ducts, when empty, are not visible. The cells of the third group, labelled *C* in the figure of Miller and Northup, were not observed.

Bodies of normally emerged cercariae, fixed without pressure in swirling, hot Duboscq-Brasil fluid and stained with Semichon technique, measure 0.135 to 0.145 mm. in length and 0.046 to 0.053 mm. in width. The tails are 0.220 to 0.240 mm. in length and 0.016 to 0.020 mm. wide at the base. The acetabulum is 0.023 to 0.024 mm. in diameter; the oral sucker is slightly larger, 0.024 to 0.025 mm. in diameter; the pharynx is oval to pyriform, usually wider posteriorly, 0.01 to 0.014 mm. in diameter; and the ceca are not developed. Above and behind the acetabulum there is a mass of deeply staining germinal cells, rudiments of the reproductive organs and ducts.

Young cercariae in rediae lack spines, there are no membranous fins on the tail, the ocelli are small and the two excretory pores on the sides of the tail are clearly visible. Indeed, in very young specimens the excretory ducts are not fused in the base of the tail. In such specimens the acetabulum is situated more posteriorly, about one-fourth of the body length from the posterior end; it gradually is moved anteriad as the postacetabular portion of the larva grows faster than the anterior portion.

Normally emerged cercariae are photonegative; they swim rapidly, tail in advance, with the body bent ventrally. The body may extend to a length of 0.240

mm. with a corresponding reduction in width. The tail is subterminal in attachment; it may be extended to a length of 0.340 mm. and the tip may be protruded as a small papilla. The central portion of the tail is 0.018 to 0.025 mm. wide near the base and the lateral cuticular fins are about the same width. The lateral fins have 30 to 36 undulatory plications, the crests of which may simulate setae. The median fin is narrower than the lateral ones; it extends along the ventral side of the distal one-fourth of the tail and forward for about one-third of that distance on the dorsal side. In a fixed and stained specimen, 44 nuclei were counted in the tail. The cuticula of the body, but not of the tail, is covered with spines. There is a narrow spineless area around the mouth and then two rows of alternating peristomial spines, typically 27 in each row. Behind these collar-spines the spines are slightly recurved with broad bases, gradually becoming narrower and smaller posteriorly. There is a pair of conspicuous ocelli or eye-spots, 0.009 to 0.012 mm. in diameter, situated above the lateral ends of the commissure of the nervous system. A very large number of pigment granules surrounds each lenticular cup. When the body is extended, the ocelli are circular but when it is retracted the ocelli are oval, wider than long.

The excretory vesicle is V-shaped when filled and Y-shaped with short arms when empty. It has a definite epithelial wall and is surrounded by cystogenous cells. It is formed by the coalescence and fusion of the excretory ducts of each side, although in young cercariae the two ducts are distinct and open on the sides of the tail, some one-fourth to one-fifth of its length from the base. The ducts in the tail subsequently fuse and regress, so that in emerged cercariae there may be only lateral excretory pores at the junction of the body and tail. The collecting ducts pass forward, ventral to the ducts of the penetration glands, from the anterolateral faces of the excretory vesicle to the level of the ocelli where they turn backward and each gives off a branch which runs in front of the ocellus and divides into three capillaries that drain the three flame-cells situated in the region of the oral sucker. The recurrent tubule passes backward and gives off a second branch that divides to supply the three flame-cells in the preacetabular and acetabular area and then continues posteriad to break up into three capillaries that drain three flame-cells in the postacetabular area. There are tufts of long cilia in the collecting ducts; movement of particles can often be seen in the capillaries and collecting ducts and the emergence of fluid from the excretory pores occurs regularly. There is no reason to doubt the distal movement of fluid in the excretory system. It may be osmoregulatory but the flow of fluid undoubtedly removes nitrogenous waste products from the body.

Development of the cercariae has been studied in serial sections of infected snails. In the morula stage of cercarial embryos a cluster of somewhat larger and more deeply staining cells is visible near the middle of the embryo. These cells are germinal and eventually give rise to the gonads, the vitellaria and the reproductive ducts. But I have not been able with certainty to determine the origin of the cells that form the epithelial wall of the excretory vesicle.

Metacercaria (Figures 1 and 2)

The cercariae encyst soon after they enter the tissues of the fish. Recently formed cysts are oval, each slightly longer than the body of the cercaria, which lies in a fluid-filled cavity. As development proceeds the larva increases in size and is bent ventrally. At first the wall is thin and flexible but it thickens, hardens and becomes very tough. The cyst shown in Figure 1 from a laboratory-reared *M. menidia,* dissected six weeks after exposure to cercariae, measures 0.28 by 0.20 mm. and the wall is 0.015 to 0.020 mm. thick. The largest cyst measured 0.37 by 0.27 mm. The cysts are not strongly enclosed by connective tissue and often drop out, especially in the pharyngeal region, as dissection is made. Released from cysts, young worms are 0.30 to 0.60 mm. in length. The specimen shown in Figure 2, fixed under coverglass pressure, is 0.62 mm. long. As development proceeds, the ocelli regress, the peristomial spines attain a length of 0.025 mm. and a width of 0.004 mm. The acetabulum and oral sucker are almost the same size, about 0.090 mm. in diameter. The pharynx, situated just anterior to the bifurcation of the digestive system, measures 0.036 to 0.043 mm. in diameter. The gonads are clearly outlined and the reproductive ducts are indicated by strands of deeply staining cells. The intestine contains small spherules, 0.005 to 0.007 mm. in diameter. The excretory vesicle is filled with minute spherules about 0.001 mm. in diameter, which render the bladder opaque and give the fluid a milky appearance when expelled.

Stephanostomum dentatum

Adult (Figures 4, 5 and 6)

Although evidence is yet lacking to prove that *Cercaria dipterocerca* is the larval stage of *Stephanostomum dentatum,* morphological agreement supports the probability that the two are conspecific.

Linton (1900) gave a brief but diagnostic description and figures of this species. The worm from *Paralichthys dentatus,* described in that paper as *Distomum* sp. and represented on Plate 40, Figures 73, 74, and 75, is probably a specimen of *S. dentatum* from which the peristomial spines had been lost. Forty years later, Linton (1940) gave a record of collections and restated the diagnostic characters of *S. dentatum.* He determined the number of peristomial spines as 54, their length as about 0.050 mm.; he reported that they are nearly uniform in diameter for the basal half of their length, then taper gradually to the tip, which is sharply pointed. He noted the frequent loss of spines from both the peristomial rows and the general body-surface. He recognized that the mass of spermatozoa in the female duct is in the initial portion of the uterus, not in a seminal vesicle. The largest measured specimens were 2.80 mm. long by 0.100 mm. wide and 2.91 mm. long by 0.77 mm. wide. Specimens deposited in the Helminthological Collection of the U. S. National Museum are mounted on two slides which bear the numbers 8199 and 8200. Slide No. 8199 bears twelve specimens collected October 6, 1911, by Vinal Edwards. The largest worm is 2.31 mm. long and 0.81 mm. wide. The entire surface of the body is spined and the circumoral spines (shown in Figure 5) are clearly 54 in number. This specimen is designated as type of *S. dentatum.* The smallest worm, shown in Figure 4, is 0.70 mm. long; another, 0.94 mm. long, has no eggs in the uterus, and one 1.06 mm. long contains several eggs. The specimen shown by Linton (1900; Plate 39, Fig. 64) contains eggs. According to the legend the figure is × 100, and since it measures only 80 mm., the worm was less than 1.00 mm. long. The largest specimen collected during the present investigation, fixed under coverglass pressure and shown in Figure 6, measures 2.79 mm. in length. It is clear that the worms become gravid at a length of about 1.00 mm. and most of them when stained and mounted do not exceed 2.00 mm. in length.

The single specimen on Slide 8200, Helminth. Coll., collected September 5, 1919, presumably by Professor Linton, and figured in his (1940) paper (Plate 3, Fig. 25) as a representative of *S. dentatum,* measures 3.50 mm. in length and is shown in Figure 8 and 8a. All the spines, except for a few peristomial ones, have been lost and vestiges of the eye-spots remain. The sockets of certain of the peristomial spines are visible and the few spines which remain are broad, flat (Fig. 8a), and taper abruptly to sharp points. They are 0.045 to 0.050 mm. in length. While the over-all morphology is that of *Stephanostomum,* the vestiges of ocellar pigment indicate that the specimen is young, and two features, its size and the shape of the peristomial spines, denote that it is not *S. dentatum.* It probably represents an as yet unrecognized and unnamed species.

Linton (1905) reported *S. dentatum* from seven host species: *Coryphaena equisetis, Lophopsetta maculata, Micropogon undulatus, Paralichthys albiguttus, Paralichthys dentatus, Pomatomus saltatrix,* and *Rachycentron canadus,* at Beaufort, North Carolina. Manter (1931) listed *S. dentatum* as a common parasite of *P. dentatus* at Beaufort, but he (1947) reported that some of the specimens of Linton were not suitable for identification and that *S. dentatum* seems characteristically to be a parasite of flounders. In this paper, Manter (1947) reported *S. dentatum* from the marine fishes, *Epinepheles adsencionis, Epinepheles morio* and *Mycteroperca venenosa* at Tortugas, Florida. But the material consisted of only three specimens and a fragment that lacked both tips. He noted that in the specimens from Florida the gonads were separated and the intervals between them contained vitelline follicles, whereas in the specimens from Beaufort the gonads were close together and not separated by vitellaria. Since this appeared to be a constant character, he suggested that additional material might justify a new species for the Tortugas specimens. In the worm from *E. adsencionis,* shown in his Figure 61, the gonads are smaller and situated more posteriad than in specimens of *S. dentatum* (compare with Figure 6 in this paper). In the Florida material, the separateness of the gonads may be correlated with their smaller size, which permitted extension of the vitellaria into areas between them. The smaller size of the gonads may be due to genetic factors or to development in unusual hosts, or indeed, the worms may not be normal parasites of the fishes in which they were found; instead, they may have been ingested in other hosts. Digenetic trematodes of fishes may persist for some time in the digestive tract of predators and such a possibility should be considered when dealing with instances where the worms are irregular in occurrence or few in number.

Certain morphological details may be added to earlier descriptions. In unpressed specimens the body, especially the postacetabular region, is almost circular in cross-section. The prepharyngeal region, when elongated, is slender, which gives the oral sucker a funnel-like or bell-shaped appearance. In young specimens, before the vitelline follicles develop, the connections between the intestinal ceca and the excretory vesicle to form the uroproct are easily seen. The peristomial spines (Fig. 5) are slightly longer than 0.050 mm. The gonads are almost if not quite contiguous and frequently overlap, the ovary is situated slightly on the right side, the anterior testis sometimes slightly left. The seminal vesicle is followed by a prostatic portion of the male duct and both the cirrus and metraterm bear minute spines. The cirrus is protrusible and in one specimen measures 0.19 mm. long and 0.08 mm. wide.

Discussion

The tendency for members of *Stephanostomum* to lose cuticular spines has led to confusion and taxonomic difficulties as noted by Pratt (1916), Manter (1934) and other authors. Attempts to correlate particular larval and adult stages have not been fruitful, although the taxonomic problems, identity and number of valid species, and systematic position of the genus will not be finally resolved until life-cycle data are available.

Differences in the accounts of Martin (1939) and Wolfgang (1954, 1955a) on the life-histories of *S. tenue* and of *S. baccatum,* respectively, are perplexing. According to Martin, the cercaria of *S. tenue* has a simple tail with a sticky tip, a stenostomate excretory pattern and a flame-cell formula of $2\ (3+3+3+3+3+3+3)$. According to Wolfgang, the cercaria of *S. baccatum* lacks a stylet and peristomial spines, has a simple tail with a sticky tip, a mesostomate excretory pattern with the pore at the tip of the tail, but the flame-cell pattern was not worked out. Wolfgang noted that the cercaria of *S. baccatum* is distinctly different from that of *S. tenue*. But his observation that the excretory vesicle discharges through a duct which traverses the tail and opens at the tip is so unusual that it can be accepted only after confirmation. Moreover, his description of a mesostomate excretory pattern in the cercaria and a stenostomate arrangement in the metacercaria would necessitate a major alteration in the collecting tubules and suggests that the cercaria and metacercaria belong to different species.

Cercaria dipterocerca differs from the cercaria described by Martin as the larva of *S. tenue* in several important features. It lacks a stylet, has lateral and dorsoventral cuticular fins on the tail, and the flame-cell formula is simpler, $2\ (3+3+3)$. These differences are far greater than ordinarily encountered between cercariae of species in a single genus and present as yet unresolved problems.

The genus *Stephanostomum* has been assigned to the family Acanthocolpidae but the relationships of that family are uncertain. Most authors have included the Acanthocolpidae in the Allocreadioidea. La Rue (1957) presented a classification of the digenetic trematodes based on life-history data and designed to show genetic relationships. The system was founded primarily on the formation of the excretory bladder, supplemented by other morphological and bionomic data. The Digenea were divided into the Anepitheliocystidia and the Epitheliocystidia. The first group comprised three orders; Strigeatoidea, Echinostomida, and Renicolida, in which the excretory vesicle is thin, membranous, not epithelial at any stage, and in which the cercariae have forked or single tails and the excretory ducts extend into the tails of developing cercariae. In the second group, the excretory vesicle has a primary membranous wall which is replaced by an epithelium derived from a mesodermal cell-mass. It comprises two orders: the Plagiorchiida and the Opisthorchiida. These groups were distinguished by the location of the primary excretory pores, which in the Plagiorchiida are at the furrow between body and tail and in the Opisthorchiida on the lateral margins of the tail, near its base. The order Plagiorchiida contains two superfamilies: the Plagiorchioidea Dollfus, 1930 and the Allocreadioidea Nicoll, 1934. As noted, *Stephanostomum* has been accepted as a member of the Acanthocolpidae in the Allocreadioidea. But in *Stephanostomum* the excretory ducts extend into the tail of the cercaria, which would exclude the genus from the Allocreadioidea and require its transfer to the Opisthorchioidea. Such a disposition would require that the genus be removed from the Acanthocolpidae or the transfer of the entire family. Indeed, such an allocation has been suggested; at the annual Midwest Conference of Parasitologists, Peters reported on the development of the excretory system in an acanthocolpid cercaria and in the mimeographed abstract of the paper stated, "Thus, the Acanthocolpidae must be excluded from the Allocreadioidea and may be closer to the Echinostomatioidea, or possibly to the Opisthorchioidea." At the same meeting, Cable, Peters and Berger discussed the affinities of the Acanthocolpidae and noting substantial agreement with members of the family Campulidae, suggested possible genetic and taxonomic relationship, even inclusion in the same family. Pande (1960) erected a new genus *Brijicola* to contain a new species, *B. caballeroi*, with morphological similarities to a number of genera at present assigned to such different families as Acanthocolpidae Lühe, 1909; Acanthostomatidae Poche, 1926 emend. Nicoll, 1935; Echinostomatidae Looss, 1902 emend. Poche, 1926; Maseniidae Yamaguti, 1953; Heterophyidae Odhner, 1914; and Plagiorchiidae Lühe, 1901 emend. Ward, 1917. The genus was assigned to the Acanthocolpidae with the comment that the present concept of the family remains greatly confused and unsatisfactory.

SUMMARY

The morphology, composition and distribution of the genus *Stephanostomum* are considered. Taxonomic uncertainties and difficulties are discussed. It is postulated that the difficulties can be resolved only with knowledge of life-cycles, larval forms, and developmental stages. Previous accounts of life-histories in the genus are reviewed. The cercariae described by Miller and Northup (1926) from *Nassa obsoleta* at Woods Hole are listed and their life-cycles are traced. *Cercaria dipterocerca* Miller and Northup, 1926 penetrated and encysted in laboratory-reared specimens of *Menidia menidia*. They continued their development and the mature metacercariae manifest such precise agreement in number and shape of peristomial spines and in general morphology with juvenile and mature specimens of *Stephanostomum dentatum* (Linton, 1900) Manter, 1931, that *C. dipterocerca* may be the larval stage of *S. dentatum*. *Cercaria dipterocerca* is very different, however, from the cercariae described by Martin (1939) and Wolfgang (1954, 1955a) as the larvae of *Stephanostomum tenue* and *Stephanostomum baccatum*, respectively. The larvae described by Martin and Wolfgang differ markedly from one another. These differences are far greater than ordinarily encountered between species of a single genus and present as yet unresolved problems. The systematic position of *Stephanostomum* and the family to which it belongs are equivocal. If the genus is a member of the Acanthocolpidae, and if La Rue's

system is valid, the family must be transferred from the Allocreadioidea to the Opisthorchioidea.

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THE PARTITIONING OF BODY WATER IN OSTEICHTHYES: PHYLOGENETIC AND ECOLOGICAL IMPLICATIONS IN AQUATIC VERTEBRATES[1]

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Great strides have been made in recent years in understanding the fluid medium in which metabolic processes occur. Investigations have been largely confined to qualitative studies of the constituents of the body fluids and to the relationship of qualitative shifts to water balance or imbalance. Although water itself is the fundamental substance involved in fluid balance, it is a curious fact that, until recently, there had apparently been no attempt made to determine the apportionment of the total body water among the fluid compartments of vertebrates, except in man and a few other mammals.

Reichert and Brown (1909) and more recently Florkin (1949), among others, have ascribed both phylogenetic and ecological significance to qualitative features of body fluids. It was thought that a comparative, quantitative study of vertebrate fluid compartments might demonstrate patterns which would likewise have phylogenetic and/or ecological implications. Data have been published on the Chondrichthyes (Thorson, 1958) and on Agnatha (Thorson, 1959), and this paper presents findings on the third aquatic vertebrate class, Osteichthyes, the bony fishes.

Only six papers known to the writer have previously reported measurements of blood volume in bony fishes. These are listed in Table III, which will be treated later. Only Prosser and Weinstein (1950) have attempted measurement of the extracellular fluid volume (NaSCN space) of fish, in a single species; but no attempt has previously been made at a complete quantitative analysis of the major fluid compartments of Osteichthyes.

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TABLE I

Summary of experimental data. The mean is followed by the number of experimental animals (in parentheses). The range of measurements appears in parentheses under the mean. Standard deviation is given for plasma, extracellular fluid and total body water

	Fresh-water Chondrostei		Fresh-water Holostei		Fresh-water Teleostei		
	Lake sturgeon	Paddlefish	Bowfin	Shortnose gar	Common sucker	Carp	Bigmouth buffalofish
Weight (gm.)	3058 (8) (2275–4530)	4679 (5) (3740–5910)	1963 (6) (1020–3265)	1185 (7) (855–1730)	617 (2) (580–655)	2412 (7) (1585–3190)	3395 (8) (1980–5440)
Pulse (beats/min.)	49 (8) (44–52)	22 (5) (16–28)	20 (6) (14–28)	19 (6) (14–24)	55 (2) (47–64)	28 (6) (18–39)	38 (8) (19–70)
Respiration (per min.)	53 (8) (40–72)	17 (5) (10–26)	14 (6) (9–20)	28 (5) (24–36)	47 (2) (45–50)	27 (3) (19–38)	22 (4) (20–24)
Hematocrit (per cent cells)	22 (8) (19–29)	30 (5) (24–37)	32 (6) (32–34)	42 (7) (33–50)	39 (2) (39–40)	33 (7) (23–44)	29 (8) (18–40)
Spec. grav., plasma	1.016 (3) (all 1.016)	1.017 (3) (1.016–1.018)	1.018 (3) (1.0175–1.0185)	1.019 (3) (1.016–1.021)	1.016 (3) (1.015–1.017)	1.019 (3) (1.018–1.020)	1.016 (3) (all 1.016)
Spec. grav., blood	1.036 (3) (1.033–1.040)	1.040 (3) (1.039–1.041)	1.045 (3) (1.044–1.047)	1.051 (3) (1.050–1.052)	1.041 (3) (1.040–1.042)	1.040 (3) (1.039–1.0405)	1.042 (3) (1.041–1.043)
Plasma volume (T-1824 space)*	2.8 (8) (2.2–3.9) s.d., .50	2.2 (5) (1.8–2.3) s.d., .21	2.2 (6) (1.9–3.2) s.d., .46	2.1 (7) (1.6–2.6) s.d., .38	1.2 (2) (1.0–1.5) s.d., .25	1.8 (7) (1.4–2.2) s.d., .27	1.9 (8) (1.3–3.3) s.d., .61
Blood volume*	3.7 (8) (2.8–4.9)	3.0 (5) (2.4–3.6)	3.4 (6) (2.9–5.0)	3.8 (7) (3.0–5.2)	2.2 (2) (1.8–2.7)	3.0 (7) (2.4–3.5)	2.8 (8) (1.8–4.1)
Extracellular fluid (sucrose space)*	20.1 (8) (15.4–27.5) s.d., 3.91	15.6 (5) (14.1–16.9) s.d., .95	18.9 (6) (14.6–25.6) s.d., 3.31	13.6 (7) (12.5–14.8) s.d., .86	12.2 (2) (11.8–12.7) s.d., .45	15.5 (7) (12.0–22.1) s.d., 3.34	13.2 (8) (10.9–15.6) s.d., 1.27
Interstitial fluid (sucrose space minus plasma)*	17.3	13.4	16.7	11.5	11.0	13.7	11.3
Total body water*	72.7 (8) (71.5–74.0) s.d., .70	74.0 (5) (71.3–77.4) s.d., 2.07	74.5 (6) (72.6–76.4) s.d., 1.18	66.7 (7) (64.5–70.7) s.d., 1.85	74.4 (2) (74.1–74.8) s.d., .35	71.4 (7) (70.1–73.1) s.d., 1.19	70.6 (8) (64.1–76.0) s.d., 3.45
Intracellular fluid (total water minus sucrose space)*	52.6	58.4	55.6	53.1	62.2	55.9	57.4

* All fluid volumes expressed as per cent of body weight.

TABLE I—*Continued*

	Marine Teleostei						
	Tiger rockfish	Nassau grouper	Red snapper	Gray snapper	Green moray	Great barracuda	Rainbow parrotfish
Weight (gm.)	5885 (1)	1270 (2) (930-1610)	3765 (2) (3130-4400)	3711 (6) (1900-4680)	4062 (6) (3050-4815)	2204 (11) (1430-4575)	4607 (19) (1650-6830)
Pulse (beats/min.)	51 (1)	52 (1)	58 (2) (48-68)	54 (3) (44-66)	70 (5) (60-88)	68 (11) (32-98)	48 (16) (30-98)
Respiration (per min.)	—	—	—	41 (4) (30-48)	22 (3) (18-28)	41 (7) (30-54)	40 (14) (22-64)
Hematocrit (per cent cells)	28 (1)	28 (2) (28-29)	36 (2)	35 (6) (28-40)	26 (6) (24-28)	31 (11) (25-36)	30 (27) (20-40)
Spec. grav., plasma	Used ave. (1.017)	Used ave. (1.017)	Used ave. (1.017)	Used ave. (1.017)	Used ave. (1.017)	Used ave. (1.017)	Used ave. (1.017)
Spec. grav., blood	Used ave. (1.042)	Used ave. (1.042)	Used ave. (1.042)	Used ave. (1.042)	Used ave. (1.042)	Used ave. (1.042)	Used ave. (1.042)
Plasma volume (T-1824 space)*	2.3 (1)	1.8 (2) (1.6-2.1) s.d., .25	1.3 (2) (1.3-1.4) s.d., .05	1.3 (6) (1.2-1.5) s.d., .10	1.6 (6) (0.7-2.3) s.d., .63	1.9 (10) (1.5-2.6) s.d., .30	2.4 (16) (1.9-3.2) s.d., .42
Blood volume*	3.3 (1)	2.6 (2) (2.3-3.0)	2.2 (2)	2.0 (6) (1.9-2.5)	2.2 (6) (1.0-3.0)	2.8 (10) (2.3-3.7)	3.6 (16) (2.5-4.7)
Extracellular fluid (sucrose space)*	12.5 (1)	14.5 (2) (13.1-16.0) s.d., 1.45	14.0 (2)	14.0 (6) (12.2-15.3) s.d., .97	15.8 (6) (12.2-19.4) s.d., 2.67	15.9 (8) (11.6-19.1) s.d., 2.07	16.6 (8) (14.3-18.9) s.d., 1.60
Interstitial fluid (sucrose space minus plasma)*	10.2	12.7	12.7	12.7	14.2	14.0	14.2
Total body water*	71.1 (1)	71.7 (2) (70.4-73.1) s.d., 1.35	71.3 (2) (71.2-71.5) s.d., .15	72.3 (6) (70.5-74.0) s.d., 1.02	63.7 (6) (56.1-71.8) s.d., 5.92	70.6 (9) (68.1-73.3) s.d., 1.94	73.1 (14) (71.2-75.0) s.d., 1.20
Intracellular fluid (total water minus sucrose space)*	58.6	57.2	57.3	58.3	47.9	54.7	56.5

TABLE II

Experimental data summarized by taxonomic groups and compared with data for Agnatha and Chondrichthyes. Mean is followed by number of experimental animals in parentheses. Standard deviation given for plasma, extracellular fluid and total body water. Ranges can be determined from Table I

	Agnatha (*Petromyzon marinus*)	Chondrichthyes (summary)	Osteichthyes (summary)	Fresh-water Chondrostei	Fresh-water Holostei	Fresh-water Teleostei	Marine Teleostei
Weight (gm.)	190 (12)	3012 (65)	3195 (90)	3681 (13)	1544 (13)	2664 (17)	3710 (47)
Pulse (per min.)	31 (12)	24.5 (39)	44.5 (80)	39 (13)	20 (12)	37 (16)	57 (39)
Respiration (per min.)	122 (12)	—	35.3 (61)	47 (13)	20 (11)	29 (9)	38 (28)
Hematocrit (per cent cells)	33 (12)	18.3 (52)	30.7 (98)	25 (13)	38 (13)	32 (17)	30 (55)
Spec. grav., plasma	1.018 (12)	—	1.017 (21)	1.0165 (6)	1.0185 (6)	1.017 (9)	—
Spec. grav., blood	1.040 (12)	—	1.042 (21)	1.038 (6)	1.048 (6)	1.041 (9)	—
Plasma volume (T-1824 space)*	5.5 (12) s.d., .32	5.4 (44) s.d., 1.3	2.0 (86) s.d., .41	2.5 (13) s.d., .41	2.1 (13) s.d., .42	1.8 (17) s.d., .46	1.9 (43) s.d., .38
Blood volume*	8.5 (12)	6.6 (44)	3.0 (86)	3.5 (13)	3.6 (13)	2.8 (17)	2.9 (43)
Extracellular fluid (sucrose space)*	23.9 (12) s.d., .79	21.2 (3) s.d., 2.38	15.7 (76) s.d., 2.28	18.4 (13) s.d., 3.12	16.0 (13) s.d., 2.34	14.0 (17) s.d., 2.31	15.4 (33) s.d., 1.80
Interstitial fluid (sucrose space minus plasma)*	18.4 (12)	15.8	13.7	15.9	13.9	12.2	13.5
Total body water*	75.6 (12) s.d., .51	74.8 (42) s.d., 1.6	71.2 (83) s.d., 2.29	73.2 (13) s.d., 1.40	70.3 (13) s.d., 1.58	71.4 (17) s.d., 2.47	70.8 (40) s.d., 2.62
Intracellular fluid (total water minus sucrose space)*	51.7	53.6	55.5	54.8	54.3	57.4	55.4

* All fluid volumes expressed as per cent of body weight.

TABLE III

Blood volumes of Osteichthyes reported in the literature

Author	Species	Method	No. of specimens	Per cent body weight
Welcker (1858)	*Cyprinus tinea*	Direct (Hb)	1	1.87
Welcker (1858)	*Perca fluviatilis*	Direct (Hb)	1	1.07
Derrickson and Amberson (1934)	*Tautoga onitis*	Direct (Hb)	3	1.5
Prosser and Weinstein (1950)	*Ictalurus natalis*	T-1824	6	1.77
Martin (1950)	*Ophiodon elongatus*	T-1824	8	2.8
Martin (1950)	*Ophiodon elongatus*	Vital Red	1	1.9
Martin (1950)	*Sebastodes sp.*	Vital Red	1	2.8
Martin (1950)	Cottidae (sculpin)	Vital Red	3	2.3
Lennon (1954)	*Catostomus commersoni*	Tail severance (bleeding)	23	1.5
Schiffman and Fromm (1959)	*Salmo gairdneri*	T-1824	10	2.25

Commission; James T. Shields, Superintendent of Fisheries, South Dakota Department of Game, Fish and Parks; E. C. Saeugling, U. S. Fish and Wildlife Service Hatchery Manager, Guttenberg, Iowa; K. H. Loftus, Head, Fisheries Section, and W. J. Christie, Biologist, Division of Research, Ontario Department of Lands and Forests.

MATERIALS AND METHODS

Of the marine fish employed (all Teleostei), the great barracuda, *Sphyraena barracuda,* rainbow parrotfish, *Pseudoscarus guacamaia,* tiger rockfish, *Mycteroperca tigris,* and one gray snapper, *Lutianus griseus,* were collected from the waters surrounding the islands of Bermuda. The remainder of the gray snappers and the red snappers, *Lutianus campechanus,* were taken off the coast of Florida in the general vicinity of St. Augustine. The Nassau grouper, *Epinephelus striatus,* and green moray, *Gymnothorax funebris,* were collected off the Florida coast near the Miami area.

Of the fresh-water fish, all the teleosts were taken from Nebraska lakes or streams: bigmouth buffalofish, *Ictiobus cyprinellus,* carp, *Cyprinus carpio,* and common white sucker, *Catostomus commersoni.* Of the Holostei (fresh-water forms), the shortnose gar, *Lepisosteus platostomum,* were taken in eastern Nebraska, and the bowfin, *Amia calva,* from northeastern Iowa. Of the fresh-water Chondrostei, the paddlefish, *Polyodon spathula,* were from eastern Nebraska, and the lake sturgeon, *Acipenser fulvescens,* from the St. Lawrence River in southern Ontario.

All fish were taken in nets or traps, so were undamaged, except the lake sturgeon, which were hooked by commercial fishermen and held in a large tank for several weeks before they were used.

The fish were anesthetized with approximately 50 mg./kg. aqueous pentobarbital sodium (Nembutal) injected intraperitoneally. Respiratory movements usually continued, but the gills were irrigated artificially throughout the experiments. The anesthetic required about 30–60 minutes to take full effect, and anesthesia lasted for several hours.

Plasma and extracellular fluid volumes were measured by dilution methods modified from Keith, Rowntree and Geraghty (1915). T-1824 (Evans Blue) was employed for the former, and sucrose for the latter.

After a fish was anesthetized, a cardiac puncture was made and blood was drawn into heparinized capillary tubes for hematocrit determination. A measured quantity of blood was then drawn into aqueous potassium oxalate. The syringe was doubly calibrated to receive nine parts of blood and one part oxalate. This blood was centrifuged and the plasma was used in the blank and standard dilutions employed in colorimetry, as a control on substances normally present in plasma. A solution containing 25 mg. % T-1824 and 3% sucrose was next injected into the heart in a quantity equal to that of the blood drawn. After mixing had occurred, samples of blood were withdrawn from the heart for colorimetric determination of the dilution of the injected materials. By comparison with known dilutions, the volume occupied by the dye or sucrose could readily be calculated. However, since both T-1824 and sucrose disappear slowly from the blood, a volume calculated at one instant will be smaller than one calculated later. To obtain the theoretical concentration with complete mixing but no loss from the system, several blood samples were withdrawn over a period of time and the optical density of T-1824 and sucrose extrapolated to the time of injection (Erlanger, 1921). Equilibration of the dye was rapid, and samples were drawn at 5–10-minute intervals for about 40 minutes. Extrapolation curves showed that sucrose required about an hour to be distributed thoroughly, so less frequent samples were drawn over a period of 3–5 hours for extracellular fluid measurements. All plasma for blank, standard dilutions, and tests samples was diluted 1 : 10 with isotonic saline.

Sucrose analysis was by a modification of the method of Harrison (1942). To remove the dye complexed with plasma proteins, the plasma was deproteinized by the zinc sulphate method of Somogyi (1930). Blood glucose, which would interfere with the analysis, was removed by Little's (1949) method of heating the plasma with sodium hydroxide. Color was developed by the standard diphenylamine method, and optical density was determined with a Bausch and Lomb Spectronic 20 colorimeter at 620 mμ, as it was also for T-1824.

Whole blood volume was calculated from plasma volume and hematocrit, and total body water was determined by complete desiccation of the whole animal at 105° C.

For conversion of volume measurements to per cent of body weight, specific gravity of whole blood and plasma were required. These were determined by the method described by Todd and Sanford (1943), in which a mixture of two miscible substances (chloroform, s.g. 1.489, and benzene, s.g. .879) was adjusted to suspend a drop of the test substance. The specific gravity of the mixture was then determined by a small hydrometer, suitably calibrated and corrected for temperature.

Discussion of Results

A summary of data, by species, is presented in Table I. Each mean figure is followed in parentheses by the number of experimental animals employed. Below the mean is the range of measurements. For plasma volume, sucrose space, and total body water, the three basic fluid measurements, the standard deviation is also given. All fluid compartment measurements are expressed as percentages of body weight.

The figures for species in which only one or two animals were used cannot be regarded as reliable in view of the individual variations evident in the tabulation of ranges. Nevertheless, these have been included in the table and have been used for group comparisons in Table II. In the latter table, species have been summarized into four groups, to make possible comparisons based on habitat and major taxonomic groups. To these have been added data from previous work by the author on the sea lamprey, *Petromyzon marinus*, an agnathan (Thorson, 1959), and four species of Chondrichthyes: *Squalus acanthias*, the spiny dogfish; *Raja binoculata*, the big skate; *Raja rhina*, the long-nosed skate; and *Hydrolagus collici*, the rat-fish or chimaera (Thorson, 1958).

The paddlefish and sturgeon are members of the Chondrostei, which have a prominent, persistent notochord, a cartilaginous skeleton, and a heterocercal tail. They are generally considered the most primitive of living ray-finned fishes and, of all the species used, most closely allied to the ancestral stock. The bowfin and gar belong to the Holostei, which in general exhibit a less prominent notochord, a partially ossified skeleton, and an abbreviated heterocercal tail. They are thought to have arisen from the chondrostean line and are more advanced than the latter, but primitive in relation to the teleosts. The Teleostei include the most advanced of the fishes and are now the dominant form of life in most waters of the earth. They have a largely ossified skeleton, the least persistent notochord, and usually a homocercal tail. They are represented in this work by ten species, the buffalofish, sucker and carp from fresh water, and the remainder from salt water. Only teleosts have been used for comparison of fresh-water with salt-water forms.

Weight

Ideally, when species are compared, animals of comparable size should be used. For practical reasons concerning supply of animals, this was not possible. Martin (1950), working with *Squalus acanthias,* reported a greater blood volume in small fish than in large ones of the same species. If this phenomenon is of general application, comparative size would be a matter of great concern. Although some series of present measurements suggested the same findings as Martin's, the pattern was not consistent enough to warrant a positive statement. Species have therefore been compared regardless of size, although reservations on these grounds may be in order.

Respiratory movements

The rates of respiratory movements were determined after anesthesia and cannot be assumed to reflect accurately the respiratory activities in unanesthetized animals. Considerable diversity exists, figures ranging from 14 per minute in the bowfin to 53 in the lake sturgeon. Great diversity is evident within each of the four major groups, and no consistent pattern is discernible which can be related to taxonomic categories. Three of the primitive species have among the lowest respiratory rates (gar, bowfin and paddlefish), but the sturgeon tends to negate the conclusion that a slow respiratory rate is a primitive characteristic. The average rate of all fresh-water species, regardless of taxonomic group, is about 33, as compared with 38 for marine species. It is tempting to speculate on the ecological significance, but actually this is not a great difference in view of the great diversity within both groups. It would seem reasonable that the rate of respiratory movements would be related to metabolism, but figures are not available on metabolic rates of these species. The possibility also suggests itself that a correlation might exist between respiratory rate and general visible activity. No quantitative study was made, but general observations do not support such conclusions. It is true that the paddlefish (17 per minute) is relatively sluggish. After removal from water it scarcely struggled, and could probably be worked on without anesthesia, although this was not done. The bowfin, however (14 per minute), was far from sluggish and docile, and the sturgeon (53 per minute), although not as sluggish as the paddlefish, was far less active than most of the other species.

Pulse

The pulse rates were also taken immediately after anesthesia, so cannot with confidence be compared with rates of unanesthetized animals. The most striking observation from the present measurements is the rather close correspondence of relative pulse rate with the rate of respiratory movements. With only minor discrepancies, a relatively high rate of heart beat is found in species with a high respiratory rate. This is also apparent when the four summarized groups are compared. However, in this case, there is an appreciable difference between the average pulse rate of marine teleosts (57 per minute) and fresh-water teleosts (37) or the average of all fresh-water species of Osteichthyes, including the primitive one (32). The higher pulse rate, perhaps together with the somewhat higher rate of respiratory movements of marine fish, is very likely related to the difference in external medium and/or to basal metabolism and its visible manifestation, relative bodily activity. These data point up strongly the need for work on metabolic rates in these species. Since the gar, bowfin and paddlefish have decidedly the lowest pulse rates of all species studied, a strong case could be made for slow pulse as a primitive characteristic, but for the sturgeon, whose pulse rate of 49 is in the range of teleost rates. Even so, the average of all the Chondrostei and Holostei is 29 (fresh-water teleosts 37, marine teleosts 57) so it might be said that a slow pulse is a tendency in the primitive fishes, even though the lake sturgeon is an exception.

Specific gravity of plasma and whole blood

Specific gravities were determined only for fresh-water species, so comparisons cannot be made between species of different external medium. No correlation is evident between specific gravity of whole blood and phylogenetic position of the three groups of fresh-water fish. The differences between plasma specific gravity values for the three groups are so small as to be virtually meaningless. No published plasma values were available for marine fish. Martin's (1950) average figure for whole blood of three ling cod (*Ophiodon elongatus*) was 1.044. For purposes of calculations in the marine species, the mean values obtained for fresh-water fish were used (1.017 for plasma; 1.042 for whole blood).

Hematocrit

The per cent of cells in the blood was determined in each animal for calculation of whole blood from plasma volume. The results are in general agreement with figures given by Martin (1950) and Prosser and Weinstein (1950), although they are far above the 7% figure given by Welcker (1858), which must have been in error. There is little difference between the hematocrit of marine teleosts (30) and fresh-water teleosts (32) or all fresh-water species (31). There is no clear correlation with the phylogenetic sequence, nor with differences in pulse rate or respiratory movements. However, the differences in hematocrit of the three groups of fresh-water species correspond, in a manner that could be expected, with the differences in specific gravity of whole blood.

Plasma and whole blood volume

Techniques of plasma and blood volume determination have been debated vigorously for many years and have been discussed most recently by Gregersen and Rawson (1959). It is recognized that the T-1824 dilution method has certain limitations, but still more serious objections have been raised to many other methods. In light of published discussions, as well as for practical considerations, T-1824 was selected for earlier work by the writer (1958, 1959). For the purpose of reliable comparison, it has been used in this investigation and will continue to be used in the other vertebrate groups.

When a dye is introduced into the bloodstream it is distributed only in the fluid portion of the blood, and therefore calculation of the volume it occupies is a measure of the plasma rather than whole blood. Confusion on this point (see discussion in Gregersen and Rawson, 1959) by some early users of the dilution method resulted in some plasma volumes being published as blood volumes. As a part of a study of partitioning of body fluids, plasma volume is the pertinent measurement since the water in the corpuscles is not a part of the extracellular or vascular water, but is rather a part of the intracellular compartment. However, from the standpoint of the heart and general circulation, the whole blood volume is the important consideration. Measurements of both volumes are included here.

Plasma and blood volumes of bony fishes have long been known to be low in comparison with those of other vertebrates, but recorded measurements have been rather fragmentary. All known previous measurements concerned exclusively with Osteichthyes are included in Table III. Most of the earlier results are lower than mine. However, in the most extensive study (Martin, 1950), all individual figures were within the range of variation of individual teleosts in the present study (Table I), and the average figure (2.6% of body weight) of Martin's teleosts (all marine) was only slightly lower than my marine teleosts (2.9%) shown in Table II.

It was suggested by the writer (1959) that, among aquatic vertebrate classes, a relatively large plasma and blood volume might be regarded as a primitive characteristic. *Petromyzon marinus* (Agnatha, the most primitive class of vertebrates) has a plasma volume of 5.5% of the body weight, and blood volume of 8.5% (Table II). Chondrichthyes, also a relatively primitive class, but more advanced than agnathans, has virtually the same plasma volume (5.4%), but, in agreement with the lower hematocrit value, has a lower blood volume (6.6%). None of the species of Osteichthyes, the most advanced of the three aquatic vertebrate classes, had a plasma volume of more than 2.8% (lake sturgeon) or a blood volume of more than 3.8% (shortnose gar). The case for the phylogenetic significance of vascular volume is supported by small differences in plasma volume in the three groups of fresh-water Osteichthyes. The Chondrostei, the most primitive, averaged 2.5%, the intermediate Holostei 2.1%, and the most advanced Teleostei 1.8%. The same is evident for blood volumes except that the higher hematocrit value of the Holostei increases their blood volume (3.6%) to slightly more than that of the Chondrostei (3.5%). All four of the primitive species consistently equal or exceed all species of teleosts in plasma and blood volume, except the tiger rockfish, whose single specimen is by itself virtually meaningless, and the rainbow parrotfish. A satisfactory explanation of the latter species' relatively high plasma volume (2.4%) and blood volume (3.6%) has not been found. Sufficient specimens (16) were employed to obtain reliable results; the species cannot be considered as primitive among the teleosts on morphological grounds; its external medium is not different from that of the other marine species; comparative metabolic rates are not known, and relative activity was not determined; and Martin's (1950) size:blood volume relationship cannot account for the "discrepancy," since these fish were among the largest employed.

A comparison of fresh-water teleosts with marine teleosts reveals a striking similarity in both plasma and blood volumes, only 0.1% separating the two groups in both cases. Considering the radically different osmotic environment of the two groups and the great divergence in physiology of water balance, this is a remarkable example of phylogenetic homeostasis.

The difference between the two groups is so slight as perhaps to be meaningless, but the fact that the figures for the marine species are higher than those for fresh-water species should be kept in mind when the sucrose space is considered later.

Extracellular fluid volume (sucrose space)

The closest approximation to the extracellular fluid volume attainable with available methods is the calculation of the volume occupied by a substance which, when injected into the blood stream, will filter readily from the capillaries into the intercellular spaces. The substance must not be metabolized or excreted rapidly, and must not be extensively taken up by the cells. It should also penetrate the minor fluid compartments, such as coelomic, cerebrospinal, and ocular fluids, which are likewise extracellular. A substance which satisfies all of these demands has not yet been found, but a number of compounds have been used which appear to approach the specifications in varying degree. Among them are thiocyanates, ferrocyanides, sulfates, chlorides, and bromides, and various carbohydrates such as inulin, raffinose, and sucrose. Inorganic ions are unsuitable for extracellular fluid determinations of marine fishes, since they are excreted rapidly by the gills (Smith, 1930). This was also demonstrated with thiocyanate in marine Chondrichthyes (Thorson, 1958), so such substances were not employed on the Osteichthyes.

TABLE IV

Comparison of inulin, raffinose and sucrose spaces of Pseudoscarus guacamaia

	Vol. (per cent body wt.)	Range	Aver. wt. (grams)	No. of specimens
Inulin	11.4	9.2–14.5	5451	8
Raffinose	14.4	12.7–16.4	4096	4
Sucrose	16.6	14.3–18.9	4696	8

In my work on Chondrichthyes, the spaces penetrated by inulin, raffinose, and sucrose were compared. These were, in *Squalus acanthias*, respectively, 12.7, 15.2, and 21.2% of the body weight. These volumes are in inverse order to the molecular weights of the three substances: 990, 594, and 342. So that a comparison with at least one bony fish could be made, the same three substances were also compared in the rainbow parrotfish (Table IV), and much the same results were apparent. Sucrose appeared to penetrate more thoroughly in both groups of fish than either inulin or raffinose. Sucrose is considered by many investigators as the substance of choice for higher vertebrates (Robertson, 1953; Wilde, 1945;

Kruhøffer, 1946). It was used in this study, and for purposes of comparison will be employed in future studies on other vertebrate groups.

None of the substances employed by myself and other workers has been shown to penetrate appreciably into the minor fluid compartments. In Chondrichthyes (Thorson, 1958) coelomic, cerebrospinal, and ocular fluids were analyzed for both inulin and sucrose penetration, and the results were negative. In the present work, only the ocular fluid was of sufficient quantity to test, and sucrose was not found in it. This introduces a small error into the extracellular fluid measurements, but the cerebrospinal and coelomic fluids were so small in quantity that they could not be measured directly and it is estimated that the ocular fluid would not make up more than about one-fourth of one per cent of the body weight in any of the species employed. No correction for minor fluids has been introduced in the tables.

It should be borne in mind that the fluid reached by the sucrose includes also lymph, since this is interstitial fluid en route back into the vascular system. It has been impossible to measure lymph separately, so it is included with the interstitial fluid volume.

When the sucrose spaces of the three fresh-water groups are compared, an even more pronounced relationship in the taxonomic series appears than for plasma volume (Table II). The more primitive the group, the higher is its extracellular fluid volume: Teleostei, 14.0%; Holostei, 16.0%; Chondrostei, 18.4%. This continues to be apparent when the comparison is extended to the Chondrichthyes (21.2%) and Agnatha (*Petromyzon marinus*, 23.9%), the most primitive class having the largest sucrose space of all the aquatic classes.

It must be noted that the figure for extracellular fluid volume of the Chondrichthyes is based on only three individual measurements. These are three *Squalus acanthias*, the only chondrichthyans in which sucrose was employed. Otherwise, all extracellular fluid measurements of the four species of Chondrichthyes were made with the use of inulin, which gave lower results (see above). The average inulin space of 31 chondrichthyan fish was 11.9% of the body weight. This figure can be compared directly only with the rainbow parrotfish, in which the inulin space was 11.4% (Table IV). If the sucrose space of the rainbow parrotfish (16.6%, based on eight individuals) is increased by a factor of 11.9/11.4, the result might be used as a calculated sucrose space for Chondrichthyes. This would be 17.3%, as compared with 15.7 for Osteichthyes and 23.9 for *Petromyzon marinus*. The same gradient in extracellular fluid volume is apparent as before, although the difference between Chondrichthyes and Osteichthyes is not as pronounced.

When fresh-water teleosts are compared with marine teleosts, it is seen that the latter have a somewhat larger sucrose space (15.4%) than the former (14.0%). Considering the extreme osmotic dissimilarity of the fresh- and salt-water media, these figures may be more remarkable for their relative closeness than for the slight difference apparent. The difference which exists is probably related, among other things, to the slightly higher osmotic pressure of the blood of marine fish as compared with fresh-water fish (Prosser *et al.*, 1950; Florkin, 1949; Williams, 1951).

There is some variation within all the groups, and, as for plasma and blood volumes, the rainbow parrotfish has the largest sucrose space of all the marine teleosts, although the difference is not as great in this case. Its sucrose space is also greater than that of any fresh-water teleost and only that of the bowfin and rock sturgeon of the primitive forms exceeds it.

Only one earlier measurement of the extracellular fluid compartment in any aquatic vertebrate is known to the writer. Prosser and Weinstein (1950) determined the thiocyanate space of six yellow bullheads, *Ictalurus natalis,* which are fresh-water teleosts. The average volume was 4.0% of the body weight. In this paper, the average for all fresh-water teleosts was 14.0. The lowest species figure was for the two common suckers (12.2), and the lowest individual measurement was 10.9 for a bigmouth buffalofish. No marine teleost figure was this low, although when inulin was employed (Table IV) a figure of 9.2 was obtained for a single rainbow parrotfish. In view of the great divergence in results, it is inconceivable that the measurements of Prosser and Weinstein could represent chance variation, and it appears probable that the explanation must be sought in differences in substances employed, technique, and interpretation of extrapolation curves.

Interstitial fluid

When the extracellular fluid volume (sucrose space) and plasma volume (T-1824 space) are known, a reasonable approximation of the interstitial (tissue) fluid volume can be reached by subtracting the former from the latter. The lack of information on the minor fluid compartments does not affect this calculation, since the sucrose does not penetrate these compartments. It should be remembered, however, that the figures for interstitial fluid (Tables I and II) include the lymph.

The interstitial fluid volume could not be calculated for every animal employed, since the extracellular fluid and plasma measurements were not always both successful in the same animal. For this reason, the interstitial fluid volume for every species or other taxonomic group was derived from the mean extracellular fluid and plasma figures for that species or group. Therefore, ranges and standard deviations are not given for interstitial fluid in Tables I and II.

The most primitive group (Chondrostei) has the largest interstitial volume (15.9% of the body weight), followed by the Holostei (13.9%); the fresh-water teleosts have the lowest volume (12.2%). The marine teleosts have a somewhat higher volume (13.5%) than the fresh-water teleost species. Comparing the three classes of aquatic vertebrates, Agnatha (*Petromyzon*) has the highest interstitial volume (18.4%), Chondrichthyes has a volume of 15.8%, and the Osteichthyes 13.7%. It is seen, then, that the volume of extracellular fluid relative to the taxonomic groups is reflected in a similar fashion in both of the extracellular sub-compartments, namely plasma and interstitial fluid.

The total circulating fluid, both intra- and extra-vascular, appears to be more plentiful in primitive aquatic vertebrates than in the more advanced, and slightly more plentiful in salt-water forms than in fresh-water.

Total body water

The use of antipyrine for determination of total body water (Soberman, 1950) was unsuccessful in marine Chondrichthyes (Thorson, 1958) and was not attempted in the Osteichthyes. Complete dehydration at 105° C. was chosen as the method most practical for comparison of a wide variety of vertebrates.

In general, the total water content is fairly uniform among osteichthyan species, with two exceptions (Table I). The green moray contained only 63.7% water, appreciably below any other teleost species or the average for teleosts, either fresh-water or marine. This is almost undoubtedly due to the great quantity of oil contained in their bodies. The shortnose gar also had a lower water content (66.7%) than any of the fresh-water species. In this case, the low figure was probably related to the heavy investiture of ganoid scales.

The green moray and gar figures obscure what otherwise would be a comparative picture similar to, although less pronounced than, that for plasma and extracellular fluid. As it is, the fresh- and salt-water teleosts are very close in water content, marine forms having a little less rather than more. Holostei have somewhat less than fresh-water teleosts, even though the bowfin has the highest water content of all species measured. The most primitive group, Chondrostei, however, is true to form in having the highest content of all four groups. The Agnatha (*Petromyzon*) have the highest water content of all three classes (75.6%), as might be expected, and the Chondrichthyes (74.8) are also appreciably higher than the Osteichthyes (71.2).

Intracellular fluid

No method is known to the writer for direct measurement of the intracellular water of a whole organism, and no figures on volumes of the lower vertebrates are known from the literature. In the human the intracellular fluid compartment comprises about 50% of the body weight (Gamble, 1947). When total body water and extracellular water are known, the intracellular fluid volume can be calculated as the difference between the two. Since sucrose does not appear to become distributed in the minor fluids (coelomic, cerebrospinal and ocular), the intracellular fluid, calculated in this manner, includes the minor fluids as well. It has been pointed out that these are almost negligible, and the error thus introduced detracts little from the reliability of the results for comparative purposes.

As for interstitial fluid, the intracellular fluid volume for each group was derived from the mean figures for total water and extracellular water of that group, and ranges and standard deviations are not given in Tables I and II.

Variation between species in calculated intracellular fluid is considerable (Table I), and only when the summaries in Table II are considered is any pattern suggested. In a general way, the relationship of fluid volume to the taxonomic series is here almost exactly reversed from that evident for plasma and extracellular fluid. Of the three fresh-water groups, the two primitive ones have a smaller intracellular volume (Chondrostei, 54.8; Holostei, 54.3) than the teleosts (57.4). The marine teleosts have a smaller intracellular volume (55.4) than the fresh-water teleost species (57.4) and the two primitive classes (Agnatha, 51.7; Chondrichthyes, 53.6) have smaller volumes than the Osteichthyes (55.5).

It was seen in Table I that there was considerable difference between species in total water content, two species, especially, (green moray and shortnose gar) having a considerably lower water content than other species of their groups. This can probably be explained in terms of varying quantities of fats, scales, and other solids in the bodies of the various species. However, the fact remains that differences in dry weight will affect the fluid measurements when expressed as per cents of total body weight. A more accurate picture of water partitioning can be had if volumes are expressed in terms of total water. Accordingly, in Table V, intra- and extracellular fluid compartments and the latter's sub-compartments, plasma and interstitial fluid, have been converted and expressed as per cents of total body water rather than per cents of body weight as before.

A comparison of these expressions of body fluid volumes points even more strongly than the observations made earlier to the existence of a relationship between body fluid partitioning in aquatic vertebrates and the phylogenetic series, as well as to habitat.

When the figures for extracellular fluid, plasma and interstitial fluid are arranged in order, from smallest to largest, the order of the groups is exactly the same: smallest for fresh-water teleosts, followed by marine teleosts, Holostei and Chondrostei, in that order. Thus, these volumes are highest in the most primitive Chondrostei and lowest in the most advanced teleosts (both fresh-water and marine). The order for intracellular fluid is exactly reversed, the greatest volume being found in the teleosts and the smallest in the primitive Chondrostei. When the comparison of all fluid volumes is extended from the relatively advanced class Osteichthyes to the more primitive Chondrichthyes, in every case the progression is in the same direction as for the groups within the Osteichthyes: larger extracellular, plasma and interstitial volumes; smaller intracellular. The progression is extended again when the most primitive of the three classes, Agnatha, is compared with the others.

TABLE V

Body fluid measurements expressed as per cent of total water

	Agnatha (*Petromyzon marinus*)	Chondrichthyes (summary)	Osteichthyes (summary)	Osteichthyes			
				Chondrostei	Holostei	Fresh-water Teleostei	Marine Teleostei
Total water	100	100	100	100	100	100	100
Intracellular fluid	68.4	71.6	78.0	74.9	77.3	80.4	78.3
Extracellular fluid	31.6	28.3	22.0	25.1	22.7	19.6	21.7
Plasma	7.3	7.2	2.8	3.4	2.9	2.5	2.6
Interstitial fluid	24.3	21.1	19.2	21.7	19.8	17.1	19.1

It will be noted that the marine teleosts maintain their position in relation to fresh-water teleosts and the other groups in every fluid measurement: the extracellular fluid volume as well as its subdivisions, plasma and interstitial fluid, are slightly higher in marine than in fresh-water teleosts, but lower than those of any other group of aquatic vertebrates. The intracellular fluid is slightly less than that of fresh-water teleosts, but greater than that of any other group considered.

Some of the differences shown in the tables are small and statistically questionable, and data for Agnatha are somewhat meager, being based on only one species. However, the over-all pattern which emerges appears highly suggestive. When comparisons progress in the direction of primitiveness in the whole series, or from fresh- to salt-water forms within the teleosts, the proportion of total water within the cells for use in the metabolic activities of the protoplasm appears to become less, and a greater proportion of water becomes available outside the cells for transportation of raw materials and metabolites between the cells and the external environment. The more advanced groups, and, concerning habitat, especially the fresh-water teleosts, appear to get along with a smaller amount of circulating fluid, which, then, is presumably used more effectively.

SUMMARY

1. The major body fluid compartments were measured in two species of fresh-water Chondrostei, two species of fresh-water Holostei, three species of fresh-water Teleostei and seven species of marine teleosts. These were compared with previous measurements of an agnathan species and four species of Chondrichthyes.

2. A general correlation was shown between the relative rates of respiratory movements and pulse rates, but neither of these appeared to be related to the taxonomic series. A faster pulse was more characteristic of marine than of fresh-water species.

3. Plasma volume was measured by the dye dilution method, using T-1824. Whole blood volume was calculated from plasma volume and hematocrit. A progressive reduction in plasma and whole blood volume was noted, proceeding from the primitive to the more advanced groups. This is true both among the three classes of aquatic vertebrates and also among the three groups within the Osteichthyes. These volumes were remarkably similar in fresh-water and marine teleost species, although slightly higher in the latter.

4. Extracellular fluid volume was approximated by sucrose dilution. A comparison of inulin, raffinose and sucrose spaces in one species showed most thorough penetration by sucrose and least by inulin, the volumes being in inverse order to the molecular weights of the substances. Sucrose did not penetrate the minor fluid compartments (coelomic, cerebrospinal and ocular fluids) so these are not included in the extracellular data. The minor fluids probably do not exceed one fourth of one per cent of the body weight in any species studied. The sucrose space (extracellular fluid volume), like plasma volume, was found to be greatest in primitive forms and least in the most advanced. The fresh-water and marine forms have similar extracellular volumes, although slightly greater in the latter.

5. Interstitial (tissue) fluid was estimated by subtracting plasma from extracellular fluid. Exactly the same relationship with the taxonomic series and habitat obtained here as for plasma and extracellular fluid.

6. Total body water was measured by complete desiccation at 105° C. It was found to be fairly uniform among osteichthyan species, with two major exceptions, probably related to high oil content of the body and a heavy investiture of scales. The correlation of water content with the taxonomic series is not as clear in this case, although there is a greater water content in the Chondrostei than in the other two groups of Osteichthyes, and in the Agnatha and Chondrichthyes than in

Osteichthyes. Marine species of teleosts have a slightly smaller total water content than fresh-water species.

7. Intracellular water was calculated by subtracting extracellular fluid from total body water. The relationship of this compartment to the taxonomic series is almost exactly reversed from that of plasma and extracellular fluid: a relatively small intracellular volume is characteristic of primitive groups and a larger volume of more advanced groups. Marine teleosts have a somewhat smaller intracellular volume than fresh-water teleosts, likewise a reversal of the relation in plasma and extracellular fluid.

8. The phylogenetic and ecological patterns become somewhat more distinct when the volumes are expressed as per cents of total water rather than of body weight.

9. Some of the differences shown are small and statistically questionable, and comparisons involving Agnatha are based on only one species. However, the over-all pattern suggests that, in general, the more advanced forms, as opposed to the primitive, and the fresh-water teleosts, as opposed to the marine teleosts, function with a smaller proportion of mediating fluid and relatively more protoplasmic water. The circulating fluid thus appears to be utilized more effectively in the advanced aquatic vertebrates and, to a lesser degree, in fresh-water than in marine forms.

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THE EFFECT OF TEMPERATURE ON THE GROWTH AND SURVIVAL OF SEVERAL MARINE ALGAL SPECIES

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The importance of temperature as an ecological factor limiting the distribution of species is well known. Protozoa and algal species have been found in extreme temperature ranges and have been considered among the most resistant of all organisms. Flagellates have been found living in hot springs at 51° C., algal species at 70° C. to 80° C. and even as high as 90° C. (Davenport, 1897). These examples are interesting, but exceptional, and active stages of "protists" are usually killed as the temperature approaches 45° C. (Hall, 1953). Although information is available on the general characteristics of the biothermal range for many organisms, specific information on the temperature tolerance of individual species frequently is lacking. Such information as is available has often been obtained from nature where the temperature of the natural habitat is subject to fluctuations and, hence, observations may be unreliable (Spector, 1956). Information gained from laboratory studies under defined conditions is an aid in the interpretation of ecological data, just as ecological data may guide laboratory experimentation.

From a practical point of view, interest in the culture of algal and protozoan organisms as food sources for metazoan forms (Gibor, 1956; Davis and Guillard, 1958; Provasoli *et al.*, 1959) makes it desirable to accumulate detailed information on the factors necessary for the survival and growth of particular organisms *in vitro* and *in vivo*. Studies on the temperature characteristics of plankton species are a prerequisite to proposed studies on the temperature tolerance of bivalves (Davis, personal communication). The following is a report of an investigation into the biothermal range of several species of marine plankton organisms that are of importance to filter-feeding animals.

MATERIAL AND METHODS

Temperature requirements of representatives of three classes of phytoplankters were investigated.[1] *Monochrysis lutheri* and *Isochrysis galbana* of the class Chrysophyceae were used, as was *Phaeodactylum tricornutum*, also considered a chrysophyte by Hendey (1954), but which has been used by several workers as *Nitzschia closterium* (Ehrenberg), Wm. Smith forma *minutissima*, (Allen and Nelson, 1910). Lewin (1958) gives the most recent discussion of the taxonomy of *Phaeodactylum tricornutum* and considers it a chrysophyte of the order Bacillariales and proposes a new suborder with *P. tricornutum* as the only known species. Representatives of the class Chlorophyceae included *Dunaliella euchlora*,

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[1] The organisms used were in bacteria-free culture, and were from the same cultures used by Davis and Guillard (1958). Source data are, therefore, not repeated here.

Platymonas sp., *Chlamydomonas* sp. of the order Volvocales; *Chlorococcum* sp., *Chlorella* sp. of the order Chlorococcales; and *Protococcus* of the order Chaetophorales. Temperature requirements of the pennate diatoms, *Nitzschia laevis*, *Amphiprora* sp. and *Amphora* sp., and of the centralean diatom, *Actinocyclus* sp., were also investigated.

Glass-covered, 25-milliliter Fernbach flasks were used as culture vessels. These flasks allow for complete illumination and an adequate surface-volume relationship. Each vessel contained 10 ml. of medium and was inoculated with 0.5 ml. of a bacteria-free unialgal stock culture. Stock cultures from which the inocula were made are maintained at 20.5° C. ± 1° C. in a light room. The basal medium consisted of the following: KH_2PO_4, 20 mg.; thiamine HCl, 0.4 mg.; biotin, 2 μg.; vitamin B-12, 2 μg.; $NaNO_3$, 150 mg.; ferric sequestrene (13% Fe), 10 mg.; NH_4Cl, 50 mg.; $CuSO_4 \cdot 5H_2O$, 0.019 mg.; $ZnSO_4 \cdot 7H_2O$, 0.044 mg.; $CoCl_2 \cdot 6H_2O$, 0.022 mg.; $MnCl_2 \cdot 4H_2O$, 0.360 mg.; $Na_2MoO_4 \cdot 2H_2O$, 0.0126 mg.; sea water, 1 liter (22 to 27 ppt). NH_4Cl was eliminated from the basal medium for *I. galbana*.

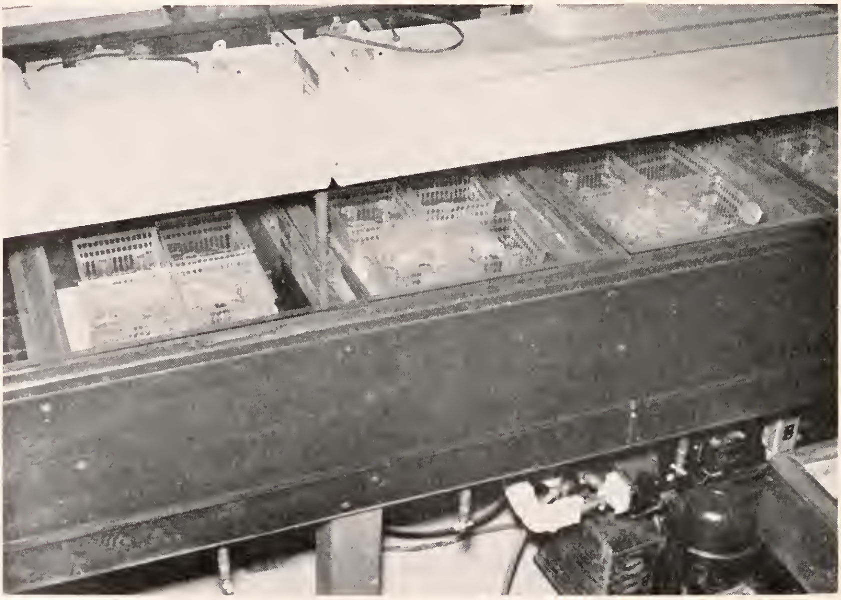

FIGURE 1. Constant temperature apparatus, showing four of the six individual baths, the refrigerating system, the lighting system and the culture vessels.

Experimental temperatures were maintained in a series of constant temperature baths regulated to ± 0.5° C. The culture flasks were submerged on plastic mesh racks into the water baths and incubated at temperatures ranging from 8° C. to 39° C. The apparatus allows for the determination of the effects of six temperatures simultaneously. Light was received from a bank of cool fluorescent bulbs suspended over the water bath to give maximum illumination with a minimum amount of heat (Fig. 1). At periodic intervals cultures were observed for multiplication of organisms. Where no growth occurred, tests for viable organisms were made by transferring a small inoculum to the basal medium, incubating at an optimum temperature and observing whether multiplication was resumed. In other experiments to determine the effect of temperature on the rate of growth and population density, cultures were incubated in loosely capped calibrated screw-cap test tubes, and growth measured as optical density in a Beckman Model DU Spectrophotometer with test tube adapter assembly.

RESULTS

All of the organisms tested grow normally within the temperature range from 18° C. to 22° C., but there was considerable variation between species, in the minimum and maximum temperatures tolerated (Table I). The maximum temperature for normal growth of the three species of chrysophyte organisms in an eight-day incubation period was 24° C. to 27° C. A temperature of 27° C. resulted in poor growth of *P. tricornutum* and *M. lutheri* and no multiplication of *I. galbana*. At 29° C., *M. lutheri* and *P. tricornutum* also showed no growth. It appeared that *M. lutheri* and *P. tricornutum* cells incubated at 30° C. and *I. galbana* at 27° C. were no longer viable, since growth was not resumed when cultures were transferred to a temperature of 20.5° C. for eight days (Table II). However, with longer incubation periods (25 days) at a favorable temperature, *M. lutheri* cells from a culture that had been incubated at 30° C. did resume normal growth. Similarly, although cells of *I. galbana* incubated at 27.5° C. showed no growth after seven days at an optimum temperature, normal multiplication was resumed by the twenty-fifth day. However, cells incubated at 30° C. did not resume growth.

TABLE I

The growth response of plankton organisms at various temperatures (degrees centigrade)

	No growth	Growth less than control	Growth equal to control at 20.5° C.	Growth less than control	No growth
M. lutheri	8–9°	12°	14–25°	27°	29–35°
I. galbana	8–9°	12°	14–22°	24–25°	27–35°
P. tricornutum	—	—	8–24°	27°	29–35°
D. euchlora	8–9°	—	12–35°	—	39°
Platymonas sp. (#1)	—	8–9°	12–32°	—	35°
Chlamydomonas sp. (Y)	8–9°	—	14–28°	—	32–35°
Chlamydomonas sp. (11/35)	—	8–9°	14–27°	—	—
Chlorella sp. (#580)	8–9°	12°	14–35°	—	—
Chlorella sp. (UHMC)	8–9°	12°	14–29°	—	32–35°
Chlorococcum sp.	—	—	15–30°	—	—
Protococcus sp. (T3)	8–9°	—	12–25°	—	26–32°
Protococcus sp. (T9)	8–9°	—	12–32°	—	35°
Actinocyclus sp.	—	15–17°	18–27°	30°	—
Amphiprora sp.	—	—	8–30°	—	—
N. laevis	—	—	15–24°	27°	30°
Melosira sp.	8–9°	14°	15–24°	—	27–30°
Amphora sp.	—	—	8–30°	—	—

Microscopic observations indicated that *I. galbana* were most actively moving

TABLE II

Tests for viability at temperatures that inhibited growth. (+ = growth equal to that of controls, ± = growth less than controls, 0 = no growth)

	Initial incubation temperature ° C.	Growth after 7 days	Growth 7 days after transfer to 20.5° C.
Platymonas sp. (#1)	7.5	±	+
	35	0	±
Chlamydomonas sp. (11/35)	7.5	0	+
	35	0	+
D. euchlora	7.5	0	+
	39	0	0
Protococcus sp. (T9)	7.5	0	+
Protococcus sp. (T3)	7.5	0	+
	32	0	0
	39	0	0
Chlorella sp. (580)	7.5	0	+
Chlorella sp. (UHMC)	7.5	0	+
	32	0	0
M. lutheri	7.5	0	+
	30	0	0
	32	0	0
I. galbana	7.5	0	+
	27.5	0	0
	30	0	0
P. tricornutum	28	0	+
	30	0	0
	32	0	0
Melosira sp.	27	0	0
	30	0	0
N. laevis	30	0	±

at 15° C. At 24° C. to 25° C. organisms appeared sluggish and pale, and at 27° C. cells settled out of suspension and only an occasional complete motile cell was observed. *M. lutheri* appeared to be most active at 18° C.; at 27° C., cells seemed sluggish and at 30° C., most of the cells were fragmented and only an occasional complete motile cell was observed.

When subjected to a temperature of 39.5° C., *M. lutheri* and *I. galbana* lost motility within 10 minutes, whereas the only motile chlorophyte tested, *D. euchlora*, remained motile for 45 minutes. In observations made five days after return to a favorable temperature, it appeared as if the viability of *M. lutheri* was affected within 20 minutes and that of *I. galbana* and *P. tricornutum*, within 10 minutes

(Table III). However, observation 13 days after return to normal temperature showed that there was some growth in all *M. lutheri* cultures, even those exposed to 39.5° C. for 65 minutes, in *P. tricornutum* exposed for 35 minutes and in *I. galbana* for 15 minutes.

The Chlorophyceae, as a whole, were more resistant to elevated temperatures than the Chrysophyceae. Of the motile Chlorophyceae, *D. euchlora* was the most temperature-resistant, *Platymonas* sp., the next, and *Chlamydomonas* sp., the least resistant. *D. euchlora* cells, exposed to 39.5° C. for 65 minutes, resumed multiplication within a few days after return to 20.5° C. (Table III), although they showed no growth during the seven-day incubation period at 39° C. (Table II). One strain of *Protococcus* sp. (T9) seemed to be more temperature-resistant than another strain (T3). *Protococcus* sp. (T9) grew at 32° C. but strain (T3) was inhibited at 27° C. (Table I). *Chlorella* sp. (580), *Chlorococcum* sp. and *Protococcus* sp. (T9) were not affected by a 65-minute exposure to 39.5° C.

Growth of the diatoms, *Amphiprora* sp., *Amphora* sp. and *Actinocyclus* sp., was not appreciably affected by temperatures between 8° C. and 30° C., but *N. laevis* and *Melosira* sp. did not grow at 30° C.

Platymonas sp. and *Chlamydomonas* sp. (11/35), incubated for seven days at 35° C., and *N. laevis* incubated at 30° C., were capable of resuming growth when

TABLE III

Thermal inactivation and death times at 39.5° C. as shown by loss of motility and failure to resume growth when returned to 20.5° C. (+ = growth equal to that of controls, ± = growth but less than that of controls, 0 = no growth)

Minutes at 39.5° C.		*M. lutheri*	*I. galbana*	*D. euchlora*	*P. tricornutum*	*Chlorella* sp. (580)	*Chlorococcum* sp.	*Protococcus* sp. (T9)
0**	% motile	100	100	100	*	*	*	*
	growth†	+	+	+	+	+	+	+
5***	% motile	50	25	100	*	*	*	*
	growth†	+	+	+	+	+	+	+
15	% motile	0	0	100	*	*	*	*
	growth†	±	0	+	0	+	+	+
25	% motile	0	0	75	*	*	*	*
	growth†	0	0	+	0	+	+	+
35	% motile	0	0	5	*	*	*	*
	growth†	0	0	+	0	+	+	+
55	% motile	0	0	0	*	*	*	*
	growth†	0	0	±	0	+	+	+
65	% motile	0	0	0	*	*	*	*
	growth†	0	0	±	0	+	+	+

* Species non-motile.
** Controls-returned to 20.5° C. immediately.
*** Time required for cultures to reach 39.5° C.
† Determined 5 days after return to 20.5° C.

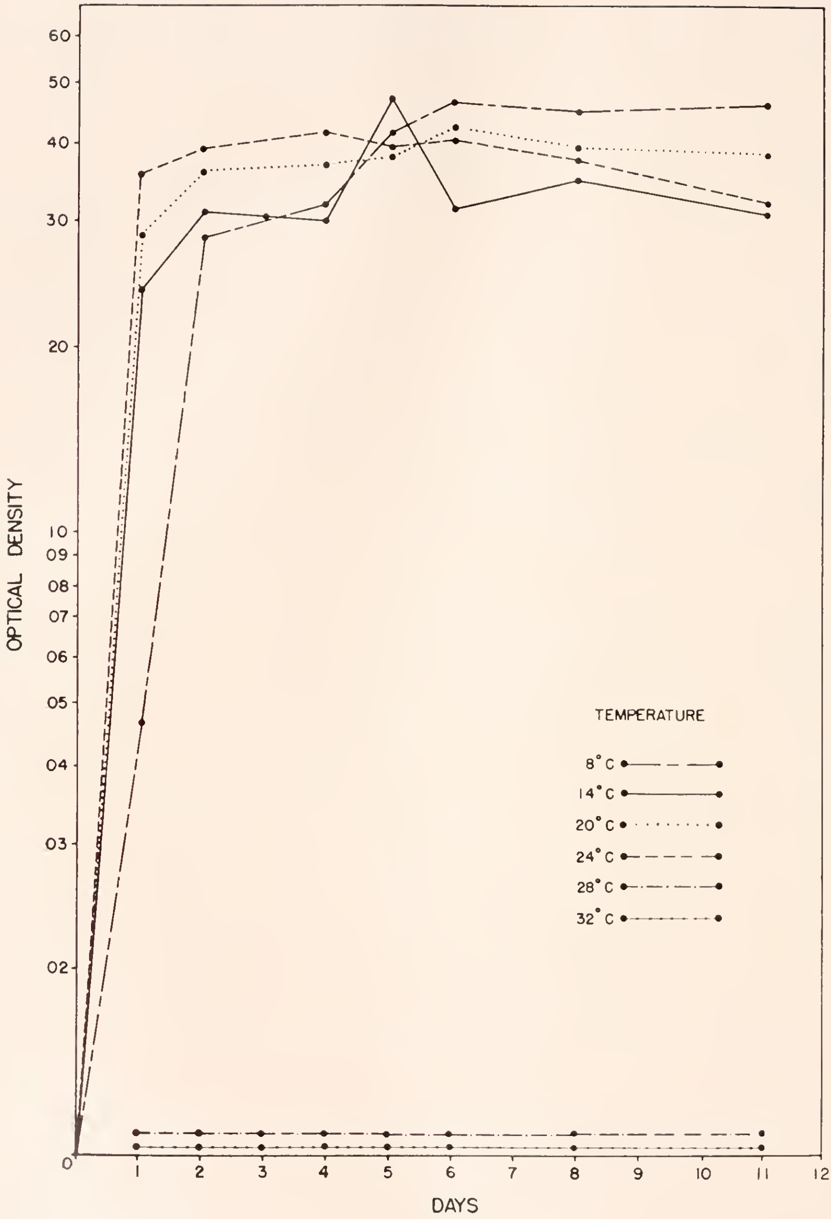

FIGURE 2. The effect of incubation temperatures of 8° C. to 32° C. on the growth of *Phaeodactylum tricornutum.*

transferred to 20.5° C. After a similar incubation period, *D. euchlora* at 39° C., *Protococcus* sp. (T3) at 32° C. to 39° C., *Chlorella* sp. (UHMC) at 32° C. and *Melosira* sp. at 30° C. were apparently not capable of resuming growth when transferred to 20.5° C. (Table II).

In every case in which growth of a species was inhibited by low temperature, growth was resumed when organisms were returned to 20.5° C. (Table II).

Quantitative measurements of the rate of growth and final population levels attained by *P. tricornutum* and *Chlorella* sp. (580) showed that *P. tricornutum* grew significantly faster at 14° C. than at 8° C. but that there was little difference in rates of growth at temperatures from 14° C. to 24° C. The maximum popula-

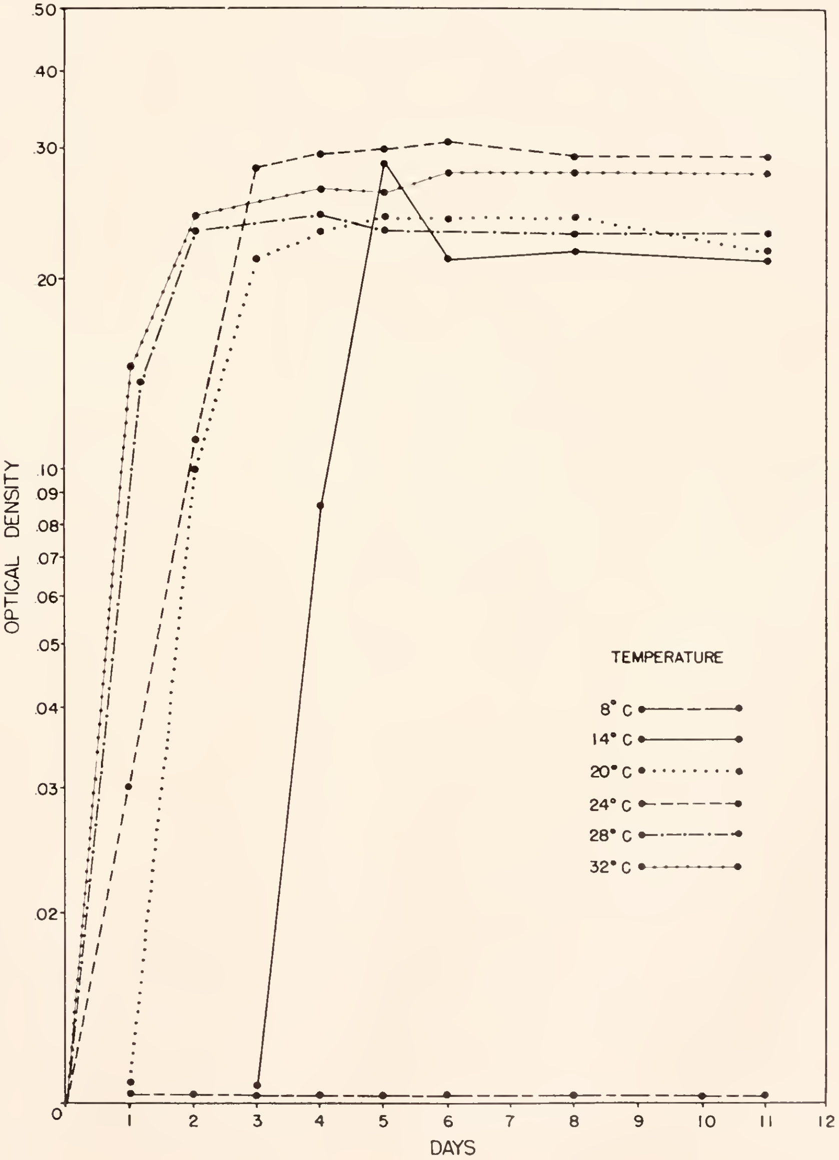

FIGURE 3. The effect of incubation temperatures of 8° C. to 32° C. on the growth of *Chlorella* sp. (580).

tion level attained, before growth leveled off, increased with each increase in temperature from 8° C. to 24° C., but no growth occurred at temperatures of 28° C. or 32° C. (Fig. 2). *Chlorella* sp. (580), conversely, showed no growth at 8° C. but grew rapidly at all temperatures from 14° C. to 32° C. At 14° C. and 20° C. initiation of growth appeared to be delayed and the maximum population level was achieved at 24° C. (Fig. 3).

DISCUSSION

For the chrysophyte organisms tested the upper temperature limits for rapid growth appear to be relatively low, between 24° C. and 27° C. It has been reported that for *Prymnesium parvum,* also a chrysophyte, the upper temperature limit for three strains was 21° C. (Shilo and Aschner, 1953). It may be that this low temperature tolerance is a characteristic of the class. Droop (1953) isolated *M. lutheri* from pools with midday temperatures of 26° C. to 27° C. but, of course, this was not a continuous temperature. Our experiments show that a few individuals of an *M. lutheri* population are capable of surviving at 30° C. and multiply when returned to favorable temperature.

It was found that the maximum temperature for multiplication of *I. galbana* is about 2° lower than that for *M. lutheri* and that the latter species is also more temperature-resistant. In an eight-day incubation period, growth of *I. galbana* was poor at 24° C. and no growth occurred at 27° C. From visual observation, growth appeared to be best at 18° C. to 20° C. Kain and Fogg (1958) found that the optimum temperature for *I. galbana* was between 20° C. and 25° C. and, although they found that there was no growth at 30° C., exposure at this temperature was not lethal. In our experiments we found that cells incubated for eight days at 30° C. did not appear viable even when cells were transferred to an optimum temperature for a period of 25 days. The difference in results may stem from two sources. The English investigators used for their experiments a culture acclimated to an incubation temperature of 25° C., whereas our stock cultures are kept at 20.5° C. ± 1° C. In addition, these investigators used a natural sea water enriched with a soil extract as an incubating medium. The addition of particular crude factors may also alter the maximum tolerated temperature. Recent work has shown that temperature tolerance in the upper temperature regions for flagellates is variable, depending upon the nutritional components of the medium (Hutner *et al.,* 1957). The latter workers showed that, at an elevated temperature, there may be a rise in the requirements for particular vitamins, trace metals or crude factors.

Although the maximum temperature of a marine environment is considered 30° C. to 36° C. (Spector, 1956), the temperature of a particular area may reach higher values, since it is dependent on the absorption of light by an algal mass, or by the depth of a shallow tide pool. Survival of organisms in nature, where high temperatures may be reached, could also be dependent upon the organic content of the water and, thus, the work of Hutner *et al.* (1957) puts a new light on the interpretation of ecological data. Temperature data obtained in different laboratories must also take into consideration the nutritional milieux as well as the past history of the organism. It may be that organisms acclimated to elevated temperatures will have a higher temperature resistance than other strains maintained at lower temperatures.

Time is also an important factor in determining the biothermal range for a "protist." The end point assay is subject to criticism, since it is dependent upon the survival of an individual organism and an analysis for this variant is being made. The temperature range for growth is narrower than that for survival.

Various theories have been proposed to account for the thermal death of an organism. These include an increase of internal protoplasmic viscosity, vacuolization of the protoplasm due to a release of calcium ion, denaturation of proteins or action on the lipid components of the cell (Heilbrunn, 1952), as well as a nutritional starvation (Hutner *et al.*, 1957). A clue to the nature of thermal death in these organisms may be furnished from studies on their suitability as foods for nutritionally-fastidious metazoan species. Loosanoff (1959) used a mixture of phytoplankton, consisting largely of *Chlorella* sp., to feed *Venus mercenaria* larvae at temperatures of 18° C. to 30° C. with satisfactory results. Experiments show that *Chlorella* sp. is not appreciably affected by a temperature of 30° C. However, in experiments using a mixture of *M. lutheri* and *I. galbana* as food, growth of larvae occurred only up to a temperature of 24° C.; at 27° C., growth was erratic (Davis, personal communication).

This inability of larvae to utilize these foods at temperatures above 27° C. may be due to the fragmentation, clumping and settling out of the food organisms. There is evidence to indicate that physical characteristics of plankton are significant in the utilization as food (Davis and Guillard, 1958). Another interesting possibility is that for the growth of bivalves there may be a nutritional factor or factors provided by the flagellates, that are heat-labile. Because the temperatures are similar, at which growth of larvae and growth of flagellates are inhibited, the possibility suggests itself that it is the heat inactivation of the same nutritional component that is the limiting factor for the growth of the flagellates as well.

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FOOD OF THE SEA-STAR ASTROPECTEN ARTICULATUS[1]

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It has long been recognized that most sea-stars are carnivorous, devouring whatever living and dead animals they come upon (Hyman, 1955). They have interested biologists because of their depredations on beds of oysters, clams, and mussels in many parts of the world. This concern has centered on the Asteriidae and other asteroids that possess suckers on flexible arms; in these forms, the prey is held or opened by the arms and digestion begins with the stomach everted. The mechanism by which such starfishes successfully attack bivalves has received much attention.

Sea-stars that do not possess suckers on their podia, or long flexible arms, must rely on swallowing their food whole and digesting it internally. This method of feeding is employed by sea-stars of the genus *Astropecten* (Hyman, 1955). Most members of this widespread genus live more or less buried in sandy bottoms, where they ingest large numbers of sand-dwelling animals. Eichelbaum (1910) and Kisch (1958) have reported on the food of *Astropecten irregularis* from northwestern Europe, Hamann (1889) recorded the food of *A. auranciacus* from the Mediterranean, and Caracelles and Parodiz (1938) reported on molluscs recovered from the stomach of *A. cingulatus* from the Argentine coast. A large portion of the food of these species consists of shelled molluscs. Because organisms are swallowed whole and their shells remain after the soft parts have been digested, the stomach contents of such sea-stars can be analyzed to determine what species have been devoured.

This report discusses the food of *Astropecten articulatus*, a common species off the southeastern coast of the United States.

MATERIALS AND METHODS

Through the cooperation of local fishermen, 124 sea-stars were obtained from the trawler "MITZI KAY" which operates from Hatteras, North Carolina. They were collected at 4 to 7 fathoms from a sand bottom near Ocracoke Inlet, North Carolina, on January 13, 1959.

The sea-stars were identified as *Astropecten articulatus* (Say) after a careful study of specimens and taxonomic literature dealing with this genus. The purple and orange coloration of living specimens gradually faded on exposure to preservative fluids or light. Because the distribution of spines on the supramarginal plates is of primary importance in the separation of species in this genus, the virtual absence of any supramarginal spines made their identification difficult. However,

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another collection from the same area in late June revealed supramarginal spines on a majority of specimens, in a confusing variability of position. It is well recognized that *A. articulatus* is a variable species, and that supramarginal spines usually develop with increasing age in this group (Verrill, 1915; Döderlein, 1917).

To expose the stomach and its contents, the oral surface was opened by separating the plates along the ambulacral grooves. The contents, often including the thin stomach wall so as to collect any small shells hidden in its folds, were carefully removed. Usually the stomach contents included a sizeable amount of sand, as well as small gastropods, pelecypods, and other animals. These were separated and sorted with the aid of a binocular dissecting microscope.

Extensive use has been made of the mollusc collection of the United States National Museum for comparison and identification of specimens. We wish to thank Drs. H. A. Rehder and J. P. E. Morrison for their generous assistance in the identification of molluscs, and Dr. C. E. Cutress for his assistance in locating *Astropecten* specimens and literature.

Results and Discussion

Of 124 *Astropecten articulatus* stomachs examined, only four were empty. Many contained the remains of a large number of animals. On the average, each stomach contained approximately twelve organisms. The maximum number was 53, two contained 46 each, and nine had 30 or more animals. These numbers exceed the maximum number of organisms recovered from *A. irregularis* by Eichelbaum (1910) or from *A. auranciacus* by Hamann (1889), and compare favorably with those cited by Kisch (1958) for *A. irregularis* from the coast of France. *Astropecten articulatus* is indeed a voracious predator on the inhabitants of the sand bottom community.

Many of the animals recovered were in a surprisingly fresh condition. The soft parts of recently ingested prey were frequently still intact; for example, the eyes of some gastropods could be seen through their transparent shells. Many gastropods retained the operculum, which often aided in identification. Kisch (1958) noted that certain gastropods protected by an operculum may survive more than 48 hours in the stomach of an *Astropecten*. From the stomach of *A. irregularis,* he was able to supply fresh specimens for the description of the soft parts of one gastropod (Fretter, 1956). Similarly, many organisms in fresh condition were recovered from *A. articulatus*. Some of these same species are only infrequently encountered in inshore collections. The ectoproct bryozoan, *Discoporella doma,* recovered from one sea-star still bore the characteristic, long, slender vibracula on its surface. Long-dead colonies of this stony bryozoan have been collected in shallow inshore sand in this region, but they typically lack vibracula and show the effects of considerable abrasion, as described by Maturo (1957).

A total of 91 species was represented in the stomachs examined. This aggregate contains two ectoproct bryozoans, two polychaete annelids, eleven arthropods, three echinoderms, fifty-two gastropods, four scaphopods, and seventeen pelecypods. The most abundant species were the small gastropods, *Acteocina candei, Natica pusilla* and *Olivella mutica*. Together these three species account for over 60% of the animals recorded. However, the decapod crustaceans re-

TABLE I

Animals recovered from stomachs of 124 Astropecten articulatus *off Ocracoke Inlet, N. C. January, 1959*

Species	No. of specimens recovered	No. of sea-stars involved
Ectoprocta:		
Discoporella doma (Orbigny)	1	1
Schizoporella unicornis (Johnston)	1	1
Annelida:		
Unidentifiable remains	1	1
Spiochaetopterus oculatus Webster tube	1	1
Arthropoda:		
Cumacea:		
Cyclaspis varians Calman	3	2
Ostracoda:		
Pontocypris edwardsi Cushman	3	2
Pseudocytheretta edwardsi Cushman	14	9
Sarsiella sp.	3	2
Decapoda:		
Sicyonia brevirostris Stimpson	2	2
Crangon septemspinosum Say	3	3
Pagurus annulipes (Stimpson)	1	1
Pagurus corallinus (Benedict)	3	3
Ovalipes ocellatus (Herbst)	13	13
Persephone punctata (Linné)	1	1
Pinnixa sp. juvenile	1	1
Echinodermata:		
Asteroidea:		
Astropecten articulatus (Say)	5	5
Echinoidea:		
Mellita quinquiesperforata (Leske)	15	12
Ophiuroidea:		
Ophiophragmus wurdemanni (Lyman)	1	1
Mollusca:		
Gastropoda:		
Vitrinella beani Fischer	2	2
Vitrinellidae	1	1
Caecum carolinianum Dall	14	12
Caecum cooperi S. Smith	1	1
Caecum pulchellum Stimpson	19	18
Alaba adamsi Dall	38	20
Finella cerithioides Dall	44	22
Adeorbis supranitidus Wood	12	11
Janthina globosa Swainson	1	1
Epitonium rupicolum Kurtz	2	2
Epitonium multistriatum Say	2	2
Sigatica carolinensis Dall	2	2
Sigatica semisulcata Gray	4	3
Natica pusilla Say	357	89
Anachis iontha Ravenel	5	3
Anachis obesa C. B. Adams	16	14
Anachis ornata Ravenel	8	5
Anachis sayana Rehder	1	1
Mitrella lunata Say	13	11
Nassarius trivittatus (Say)	2*	2
Oliva sayana Ravenel	1*	1
Olivella mutica Say	90	42

* Species represented only by long-dead shells.

TABLE I.—*Continued*

Species	No. of specimens recovered	No. of sea-stars involved
Terebra dislocata Say	1*	1
Niso interrupta Sowerby	2	2
Melanella subcarinata Orbigny	2	2
Rubellatoma rubella Kurtz and Stimpson	5	5
Rubellatoma elata Dall	1	1
Kurtziella limonitella (Dall)	4	4
Brachycythara biconica C. B. Adams	1	1
Mangelia atrostyla Dall	3	3
Mangelia oxia Bush	12	11
Mangelia sp. A	5	5
Mangelia sp. B	6	5
Mangelia sp. C	1	1
Mangelia sp. D	1	1
Cerodrillia sp.	2	2
Turridae	1	1
Acteon punctostriatus C. B. Adams	15	9
Rhizorus oxytatus (Bush)	5	4
Acteocina candei Orbigny	438	57
Cylichnella bidentata (Orbigny)	41	30
Turbonilla (*Turbonilla*) *nivea* (Stimpson)	1	1
T. (*Chemnitzia*) *aequalis* (Say)	17	14
T. (*Pyrgiscus*) *caroliniana* Tuomey and Holmes	1	1
T. (*Pyrgiscus*) *elegantula* Verrill	5	4
T. (*Pyrgiscus*) *areolata* Verrill	10	9
T. (*Pyrgiscus*) *interrupta* (Totten)	35	24
T. (*Pyrgiscus*) *mighelsi* Bartsch	1	1
Odostomia (*O.*) *modesta* (Stimpson)	3	2
O. (*Chrysallida*) *seminuda* C. B. Adams	4	2
O. (*Menestho*) *impressa* Say	2*	1
Cavolina longirostris Lesueur	2	2
Scaphopoda:		
Cadulus carolinensis Bush	9	7
Dentalium occidentale Stimpson	13	9
Dentalium eboreum Conrad	3*	3
Dentalium sowerbyi Guilding	5	5
Pelecypoda:		
Nucula proxima Say	1	1
Anadara ovalis (Bruguière)	1	1
Aequipecten gibbus (Linné)	2	2
Anomia simplex Orbigny	3*	3
Phacoides (*Parvilucina*) n. sp.	11	10
Bornia longipes Stimpson	21	15
Mercenaria campechiensis (Gmelin)	1	1
Chione intapurpurea Conrad	5	5
Gouldia cerina C. B. Adams	2	1
Macrocallista maculata (Linné)	4	2
Dosinia discus Reeve	3	3
Tellina americana Dall	2	2
Tellina sp.	1	1
Strigilla mirabilis Philippi	1	1
Ervilia concentrica Gould	7	5
Corbula swiftiana C. B. Adams	3	3
Pandora arenosa Conrad	14	6

covered probably represent a volume of digestible tissue equal to or greater than that contained in the above-mentioned gastropods. Legs, chelae, and entire bodies of the portunid, *Ovalipes ocellatus*, constituted the greatest portion of this decapod fraction. All species identified from *Astropecten articulatus* stomachs are listed in Table I, with the number of specimens of each and the number of sea-stars involved.

Although the echinoids and certain of the pelecypods were represented by juvenile specimens, the great majority of organisms collected from stomachs were adults, near the maximum size attained by each species. Exceptions to this generality include young sand-dollars, *Mellita quinquiesperforata* (less than 10 mm. in diameter), and juvenile clams, *Chione intapurpurea* and *Macrocallista maculata* (less than 6 mm. in length). Most of the other molluscs recovered were of a similar, minute size, being less than 6 mm. in length. Of the three most abundant species in this collection, *Natica pusilla* and *Acteocina candei* averaged about 3 mm., and *Olivella mutica* averaged about 4 mm. in length.

Several molluscs in this collection have not been taken in the inshore waters of the region. Notable examples are *Natica pusilla, Sigatica semisulcata, Adeorbis supranitidus, Alaba adamsi, Finella cerithioides* and *Acteocina candei* among the gastropods; the new species of *Phacoides, Macrocallista maculata, Pandora arenosa* and *Corbula swiftiana* among the pelecypods; and *Cadulus carolinensis* among the scaphopods.

In a few cases, indicated by an asterisk in Table I, shells were recovered which showed signs (discoloration, worn broken edges) that they were not fresh. It seems likely that these shells came from fossil or sub-fossil deposits in the area, particularly for those species which are not represented alive in the area today. This applies, for example, to *Nassarius trivittatus*, the New England Nassa, which has not been collected alive south of Chesapeake Bay. (Pearse, Humm and Wharton (1942) confused this species at Beaufort, N. C., with a form of *N. vibex*.) Evidently, *Astropecten articulatus* ingests mollusc shells whether they are occupied by the original inhabitant or not. Of course, some of these dead shells may have contained hemit crabs (*Pagurus* species) and thus have contributed to the sea-star's nutrition.

Several other species which appear to be out of place among the bottom fauna are represented. The purple sea-snail, *Janthina globosa*, and the pteropod, *Cavolina longirostris*, are typically pelagic, the former being carried about near the surface by its prey or by a foam raft of its own making, and the latter by active swimming. With death, these species fall to the ocean floor where they can be eaten by *Astropecten articulatus* or any other scavenger. In addition, a number of fish scales were contained in the stomachs. While some could have been ingested while the starfish were in the fish trawl, most fish scales had undoubtedly been picked up from the sand bottom.

Astropecten articulatus could be called cannibalistic in view of the presence in their stomachs of small specimens and fragments of the same species. Most of these had been ingested as fragments of broken sea-stars, perhaps left by the passage of other trawlers, although a few small specimens had been swallowed entire by larger starfish. Kisch (1958) noted such cannibalism in *Astropecten irregularis* off the coast of France, and the ingestion of other echinoderms is not an unusual practice in the genus *Astropecten*. According to Eichelbaum (1910), other echinoderms compose a major part of the diet of sea-stars of the genus *Luidia*.

Because the gastropod genera *Niso* and *Melanella* are recognized as ectoparasites of echinoderms, the occurrence of specimens of each raised the question of whether these species might be parasitizing *A. articulatus*. However, it should be noted that the asteroid *Luidia clathrata*, the echinoids *Mellita quinquiesperforata*, *Encope emarginata*, and *Moira atropos*, and the holothurian *Thyone briareus* are all present in this immediate area. Unless one can observe the feeding activities of these gastropods, no decision can be made as to which echinoderm species they parasitize.

Kisch (1958) listed sixteen molluscs recovered from *Astropecten irregularis* that represented new records for the area collected (Roscoff, France). The most notable novelties among the molluscs of the present collection are a new pelecypod (to be described elsewhere), and *Sigatica semisulcata*, a rare gastropod previously reported only from the west coast of Florida. Because the taxonomy of turrid gastropods is in a state of confusion, additional undescribed species may be represented in the Turridae of this collection.

Many of the molluscs represented have been treated in the account of species dredged by the United States Fish Commission steamer "ALBATROSS" in the Cape Hatteras region in the 1880's (Bush, 1885). In the publication, several species were described as new from this area. Their appearance in this collection serves as an indication of the value of including an analysis of starfish stomach contents among sampling methods for benthic organisms. From all indications, *Astropecten articulatus* appears to be a non-selective feeder, its stomach contents reflecting the faunal composition of the offshore sand bottoms frequented by it. Indeed, this species can serve the marine biologist as a convenient tool for the study of bottom fauna, as a dredge which samples indiscriminately the abundant minute animals that live in sand.

This generalization evidently applies equally well to other species of *Astropecten* that inhabit sand. If one compares the lists of food organisms recovered from the stomachs of various species of *Astropecten* in different parts of the world, a striking similarity becomes evident. There is a close correspondence in the types of molluscs represented, exemplified by the presence of representatives of the same genera, from widely separated localities. Making allowances for different interpretations of the latitude of certain genera, one finds that the principal difference in these lists of molluscs is at the species level. These specific differences are essentially geographic variations on a basic form, that reflect the geographic affinity of a particular sand-bottom fauna. Therefore, the food organisms recovered from stomachs of different species of *Astropecten* probably are comparable samples of the sand-dwelling fauna of their respective regions. The genus *Astropecten* may serve as a tool for comparative studies of sand-bottom communities in different parts of the world.

SUMMARY

The stomach contents of 124 *Astropecten articulatus* have been identified and discussed. This starfish ingests great numbers of small sand-dwelling organisms. Ninety-one species of invertebrates were recovered, of which 73 were molluscs. The gastropods *Acteocina candei*, *Natica pusilla* and *Olivella mutica* comprised

over 60% of the animals recovered. *Astropecten articulatus* apparently ingests whatever animals or shells it encounters. Because it effectively samples the fauna of offshore sand bottoms, its use is recommended as a collection tool for the study of this community.

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STUDIES ON THE SEXUAL ORGANIZATION OF THE RHIZOCEPHALA. III. THE MODE OF SEX-DETERMINATION IN PELTOGASTERELLA [1]

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All the known Rhizocephala except a few species of the family Sylonidae have been hitherto described as hermaphroditic animals. In the first of this series of papers (Ichikawa and Yanagimachi, 1958) it was reported that *Peltogasterella socialis* Krüger, which had been regarded as one of the representative species of the Rhizocephala, is not a hermaphrodite, but a female fertilized by larval cypris males.

A similar sexual organization was demonstrated in *Peltogaster paguri* Rathke and in *Sacculina senta* Boschma (Ichikawa and Yanagimachi, 1960). The sexes of these rhizocephalans are completely separate. The mode of sexual determination in the early stages of *Peltogasterella socialis* Krüger is discussed in this paper.

According to Delage (1884) and Smith (1906), the cypris larvae of *Sacculina* which affix themselves to the host-crabs do not differ morphologically from the larval males that attach around the mantle opening of juvenile parasites. Reinhard (1942) stated that all the cypris larvae of *Peltogaster paguri* are sexually undetermined and undifferentiated. The larvae which attach directly to the host-crabs develop into hermaphrodites. The larvae which fix to the juvenile hermaphrodites become males. Veillet (1943, 1945) reported two kinds of cypris larvae in *Triangulus galatheae* (Syn. *Lernaeodiscus galatheae*) which were considerably different in size. He surmised that the large cypris larvae become hermaphrodites and the smaller cypris larvae function as larval males. According to him, the larger cypris larvae developed from large eggs and the smaller cypris larvae developed from small eggs. Thus, Veillet suggested that the sexes of *Triangulus galatheae* are determined in the egg stage.

In this investigation, it will be reported that the sex of *Peltogasterella*, as the sex of *Triangulus*, is determined at the beginning of development.

MATERIAL AND METHODS

The material used in this investigation was *Peltogasterella gracilis* (Boschma) (syn. *Peltogasterella socialis* Krüger; *cf.* Reischman, 1959) collected from the shore of Oshoro Bay on the west coast of Hokkaido. In this locality the host of this rhizocephalan is almost exclusively the hairy sea-shore hermit crab, *Pagurus lanuginosus* de Haan.

Rearing of the adult and the larvae. It is generally believed that the rearing of the rhizocephalans under laboratory conditions is difficult. However, the author could rear both adult and larval forms of *Peltogasterella* without difficulty. The successful rearing of the adult form was effected by keeping the host-crabs in a healthy environment. The vessels in which the parasitized crabs were kept had a diameter of 15 cm. and a depth of 10 cm. One parasitized crab, after being detached from shell, was placed in each vessel with the level of sea water sufficient to cover the crab. Each vessel was covered with a glass plate to prevent evaporation of the water. Room temperature was not regulated. The crabs and their parasites were not very sensitive to temperature changes. The crabs survived for a long period of time without food. However, it is better to feed them pieces of mussel or clam at intervals. Food debris and the excrements of the crabs were carefully discarded. The sea water in the vessel was renewed at frequent intervals. The females of *Peltogasterella* on the crabs, under favorable conditions, remained alive for about two months. During this period they emitted several batches of nauplius larvae at regular intervals of two weeks (at 20° C.).

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The nauplius larvae emitted from the females were successfully reared in clean sea water when the surface of the water was covered with a film of cetyl alcohol. If the film of alcohol was not employed, a number of larvae adhered to the surface of the water and eventually perished. For the details of the method by which the author succeeded in rearing the larvae, readers are referred to Yanagimachi (1961).

Demonstration of chromosomes of mature eggs. The simple aceto-orcein squash technique was adopted to demonstrate the chromosomes. The procedures were as follows. (1) Detach mature females from the hosts and break the mantle and the wall of ovaries by the use of scissors or needles. (2) Collect the eggs with a pipette and stain them with aceto-orcein for 15 to 30 minutes. The aceto-orcein was prepared according to LaCour (1941). (3) Place the stained eggs on slide with a few drops of aceto-orcein, put a coverslip over the specimens, and blot excess stain away. Squash the stained larvae by exerting careful and controlled pressure on the coverslip. (4) Seal the coverslip with balsam-paraffin (1:1). The preparations thus made remained in perfect condition for months.

RESULTS

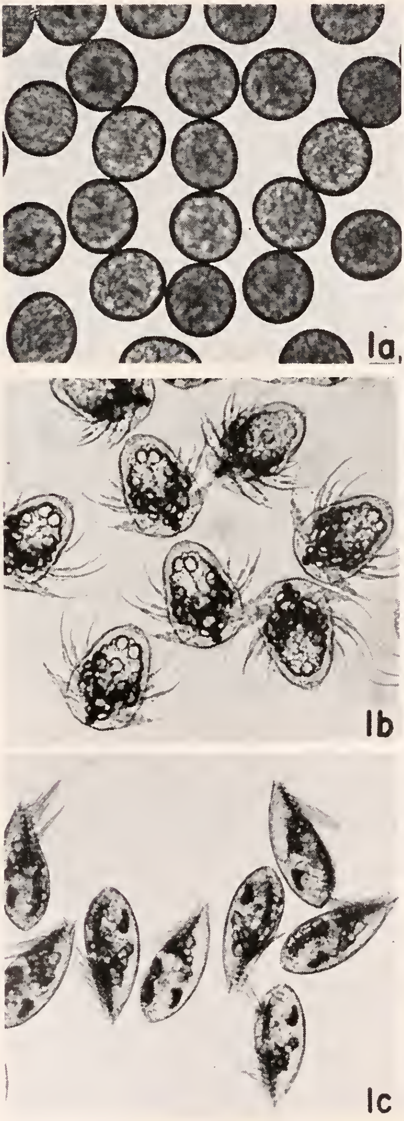
1. *The dimorphism of eggs and larvae*

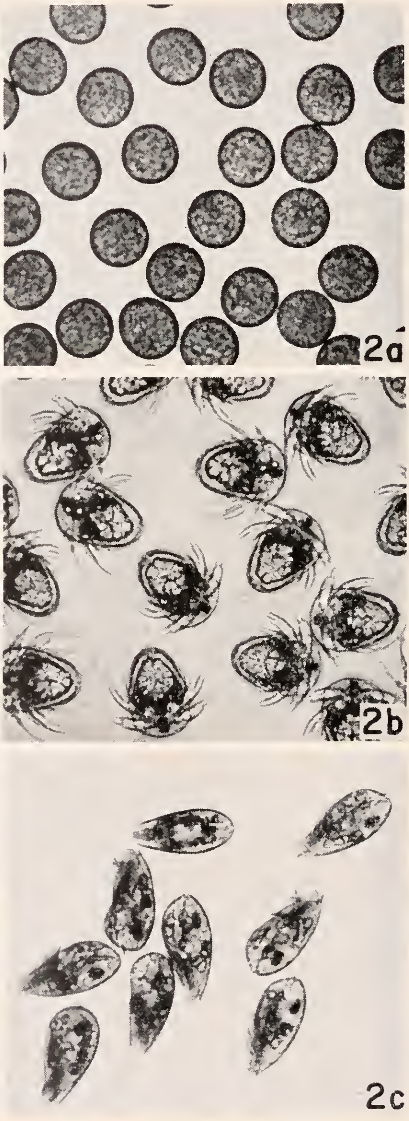
(a) When a number of adult females of *Peltogasterella* had been reared, all females on some host-crabs produced only large eggs. On the other hand, all females on other host-crabs produced only small eggs. In the maternal mantle cavity (brood pouch), these two types of eggs developed at the same rate and reached the nauplius stage after two weeks (at 20° C.). The large eggs developed into large nauplii, and the small eggs developed into small nauplii. When emitted from the maternal mantle cavity, the nauplii swam actively in sea water and underwent successive moults in 4 to 5 days. The large nauplii metamorphosed into large cypris larvae, and the small nauplii developed into small cypris larvae.

Figures 1 and 2 are photomicrographs of the eggs and larvae of the large and small types derived from the females on two different host-crabs. The measurements of these eggs and larvae are summarized in Figure 3.

The two types of eggs, *viz.,* the large and small eggs, so far as could be ascertained, did not show any distinct structural difference. They differed only in size.

In the nauplius stage, the two types of larvae were identical in structure. They were distinguishable only by their size. In the cypris stage, they were different not only in size but also in structure. The most pronounced structural difference was found in the antennules which function as organs of attachment. Figures 4





FIGURES 1a–c. Eggs and larvae of large type, derived from female *A*. (a) Mature unfertilized eggs. (b) Newly hatched nauplii. (c) Cypris. Magnification: × 55.

FIGURES 2a–c. Eggs and larvae of small type, derived from female *B*. (a) Mature unfertilized eggs. (b) Newly hatched nauplii. (c) Cypris. Magnification: × 55.

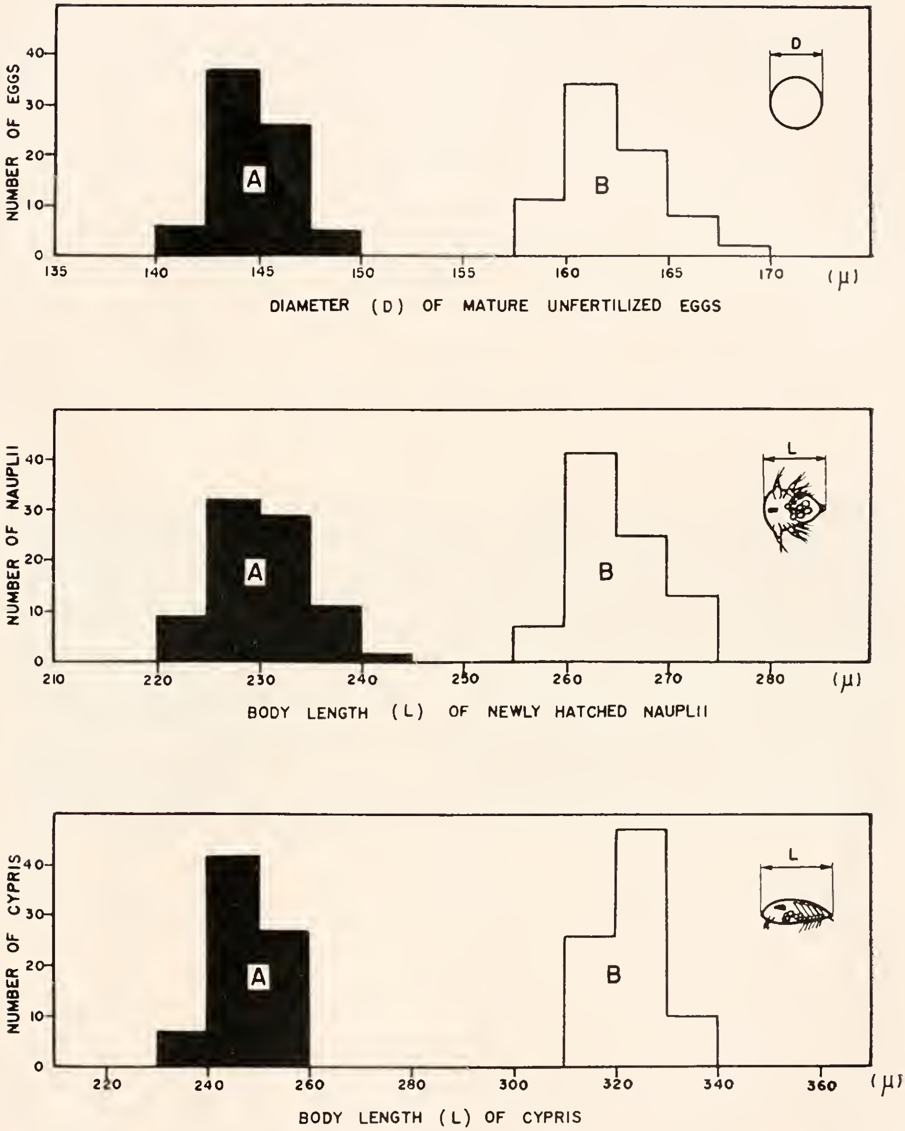


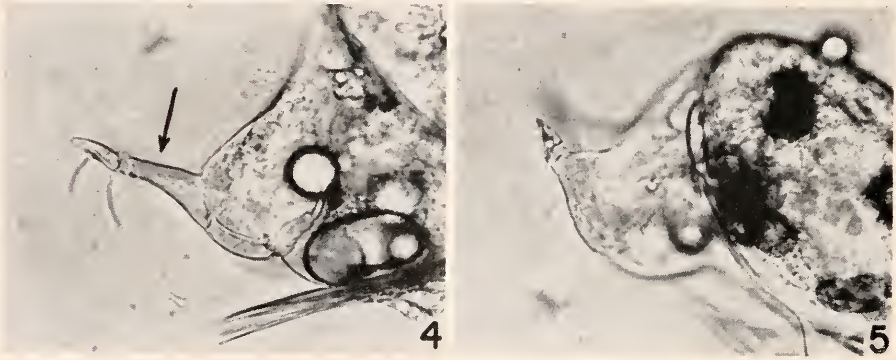
FIGURE 3. Range in size of eggs and larvae derived from two different females, *A* and *B*.

and 5 show the antennules of large and small cypris larvae. The penultimate segment of the antennule of the large cypris is comparatively long and slender (Fig. 4), whilst the corresponding part of the small cypris is greatly diminished or absent (Fig. 5).

(*b*) During its lifetime, an adult female emits several batches of larvae. Experiments were undertaken to know if one female always produces either large or small larvae.

A crab carrying adult females of *Peltogasterella* on its abdomen was placed in a vessel of sea water. All the females except one were mechanically detached. Then, the successive batches of nauplius larvae expelled from this remaining female were examined. Five experiments of this type were conducted. It was found that one female always produces either large or small larvae.

(*c*) *Peltogasterella gracilis,* in common with other species of its genus, occurs gregariously on one host. Do females of *Peltogasterella* on a single host emit both large and small larvae? Table I summarizes the measurements of the larvae and of the eggs derived from the females on six different host-crabs. It may be seen from this table that the females on one host produce larvae of one type—either large or small. The author examined the females on 25 different host-crabs. Females on 11 crabs (2 ♀ and 9 ♂) produced exclusively large larvae and those on 12 other crabs (7 ♀ and 5 ♂) produced small larvae. The females on the remaining two crabs, on the other hand, produced both large and small larvae. The data on these two exceptional females will be presented later.



FIGURES 4 and 5. Comparison of the antennules of the large and small cypris larvae. The cypris larvae were slightly compressed beneath coverslip. (4) Large cypris; an arrow indicates the penultimate segment. (5) Small cypris. Magnification: × 300.

2. *Identification of the sex of larvae*

As has been noted, Veillet (1943, 1945) found large and small larvae in *Triangulus galatheae.* He surmised that the large cypris larvae affix to the body of the host-crabs and develop into hermaphrodites, and that the small ones function as larval males. The present author came to a different conclusion about *Peltogasterella gracilis.* The experiments on which this conclusion is based are here cited.

(*Experiment 1*). On Septemeber 28, 1959, large nauplii were emitted from three females on a host-crab. These larvae were placed in a vessel of sea water and reared. They metamorphosed into large cypris larvae on October 3. On the evening of October 6, two young crabs (*Pagurus lanuginosus* of 3 mm. carapace length) were put into the vessel in which the cypris larvae were actively swimming. At the same time, a large crab (*Pagurus lanuginosus* of 10 mm. carapace length)

TABLE I

Measurements of eggs and larvae derived from Peltogasterella females on six different host-crabs

Host (*Pagurus lanuginosus*)	Parasite (*Peltogasterella gracilis*)					
Sex Carapace length	Individual	Size (length of external sac mm.)	Mean diameter of eggs (μ)*	Range of body length of nauplii (μ)**	Range of body length of cypris larvae (μ)#	Type of cypris##
Male 6 mm.	a	7	167	265–283	312–329	large
	b	7	163	250–270	312–328	large
	c	7.5	—	—	295–325	large
	d	7	—	—	312–328	large
	e	7	—	257–270	—	—
Male 11 mm.	a	7.5	161	256–276	312–336	large
	b	7	—	—	295–315	large
	c	7	—	—	312–325	large
	d	6.5	—	—	312–320	large
	e	7	—	—	311–332	large
Male 15 mm.	a	5	170	260–274	309–330	large
	b	5	168	263–268	312–329	large
	c	6	173	—	311–329	large
	d	6	—	—	312–329	large
	e	4	—	260–290	295–320	large
	f	5	—	260–276	—	—
Female 12 mm.	a	6.5	143	225–242	242–260	small
	b	7	140	219–240	225–245	small
	c	7	—	225–242	230–252	small
	d	6	—	225–242	—	—
Male 13 mm.	a	9	144	222–243	242–260	small
	b	8	140	212–234	242–250	small
	c	8.5	—	210–235	250–275	small
	d	8	—	—	242–260	small
	e	9	—	—	234–260	small
	f	6	—	—	235–255	small
Female 13 mm.	a	7.5	142	216–242	242–260	small
	b	7	145	220–240	234–256	small
	c	7	—	—	234–260	small
	d	6.5	—	207–225	—	—
	e	7	—	219–230	—	—

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* Mature unfertilized eggs which had just been released from the ovary into mantle cavity (brood pouch) were measured. The measurement was made on about 50 eggs.

** The measurement was made on 30–50 newly hatched nauplii which had not yet undergone their first moulting. The body length means the distance between the frontal margin of the carapace and the tip of the caudal spine.

The measurement was made on 30–50 recently metamorphosed cypris larvae. The body length means the distance between the anterior and the posterior ends of the carapace.

Types of cypris larvae were identified by measuring the body size and examining the structure of antennules.

carrying juvenile females of *Peltogasterella* on its abdomen was placed in the same vessel. The number of juvenile females on the crab was 15, and they varied from 2.5 to 3 mm. in length of the external sac-like portion. In every respect they were identical to those females which had recently emerged from the crab's abdomen. On the next morning, the three crabs were removed from the vessel and examined under a binocular dissecting microscope. Five cypris larvae were found attached to the mantle opening of four juvenile females of *Peltogasterella*. Two cypris larvae were about to inject their internal cellular contents into the mantle cavity of the juveniles (Fig. 6). Three cypris larvae, on the other hand, had already injected their cellular contents into the juveniles. No cypris was detected on the surface of the crab's body, although careful and repeated examinations were carried out.

(*Experiment 2*). On October 1, small nauplii were discharged from five females on a host-crab. They were placed in a vessel of sea water and reared. On October 5, they metamorphosed into small cypris larvae. On the evening of October 7, two young crabs (*Pagurus lanuginosus* of 3 to 4 mm. carapace length) were put into the vessel in which the small cypris larvae were swimming. At the same time, a large crab (*Pagurus lanuginosus* of 13 mm. carapace length), infested by 13 juvenile females of *Peltogasterella*, was put into the vessel. The next morning, the three crabs were removed from the vessel. On careful examination, several cypris larvae were found attached to the body of the young crabs. These cypris larvae were invariably affixed to the base of the setae on the crab's body. The setae on the carapace were the most frequent site of fixation (Fig. 7). No cypris was found at the mantle opening of the juvenile females of *Peltogasterella*.

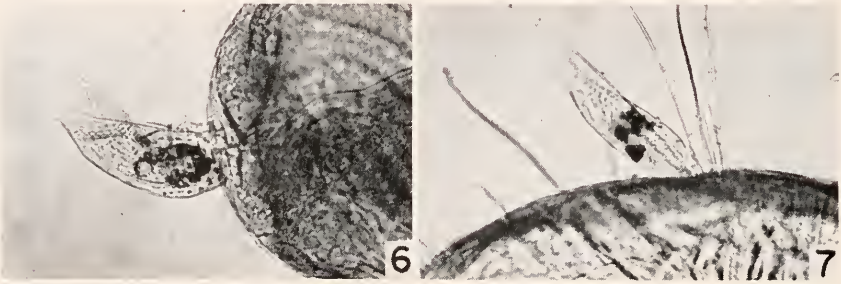

FIGURE 6. A large cypris fixed at the mantle opening of a juvenile female. Magnification: × 80.

FIGURE 7. A small cypris fixed at the base of a seta on the carapace of a host-crab. Magnification: × 80.

Fifteen experiments of these types have been conducted, with the result that the large cypris larvae always attach to the mantle opening of the juvenile females of *Peltogasterella* and never fix on the body of the host-crab. The small cypris larvae search for the host-crabs and affix at the base of setae, without being attracted to the juvenile females. From these facts it is concluded that the cypris larvae of the small type are prospective females and those of the large type function as larval males.

3. *Morphological distinction in the male-producing and female-producing females*

As stated in the first section, the females of *Peltogasterella* on one host usually produce larvae of the same type, *i.e.*, either large or small larvae. From the results of the experiments described, it is now certain that the larvae of small type are prospective females while those of large type function as males. Then, it may be said that the females on a host are regularly "female-producing" or "male-producing."

Now, a question arises as to whether the female-producing and the male-producing females are morphologically different. A number of living specimens of both female-producing and male-producing adult females were examined to obtain the answer to this question. The external appearance of the sac-like portion, the position of the stalk, the site and the shape of the colleteric glands, and the structure of the mantle opening were compared, but no distinct difference was detected. Some specimens of the female-producing and the male-producing females were cut in serial sections and examined. No discernible difference in structure of various organs of the two types of the adult females was detected. The observations of the author reveal no evident morphological difference between the male-producing and the female-producing females.

4. *The differences in the chromosomes of the male-producing and the female-producing eggs*

The eggs of *Peltogasterella* begin maturation division while they are still in the ovary. Having reached the stage of the first maturation division metaphase, the eggs are expelled through the colleteric glands into the mantle cavity of the female. The first maturation spindle then passes into telophase and the first polar body is extruded, the second polar body being immediately formed without reconstruction of the nucleus. The penetration of the sperm into the egg occurs immediately before the second polar body is extruded (Shirase and Yanagimachi, 1957).

When the author examined the metaphase plate of the first maturation division of the large male-producing eggs, 15 bivalent chromosomes were observed (Figs. 8 and 9). In the anaphase of this division, these chromosomes segregated equally and passed into the two poles (Figs. 10 and 11). In the metaphase plate of the second maturation division, 15 univalent chromosomes were counted.[3]

The chromosomal arrangements were different in the small female-producing eggs. In the metaphase plate of the first maturation division, a univalent chromosome was always found with 15 bivalent chromosomes (Figs. 12 and 13). This chromosome showed a peculiar behavior at the later stage of the first maturation division. It remained in the middle of the spindle after the other chromosomes had passed to the pole (Figs. 14–16). It was greatly stretched in the axis of the spindle and had the appearance of a body under considerable tension. In most cases it showed a conspicuous longitudinal split which was so distinct that the halves resembled two parallel lagging chromosomes. Only in the late anaphase of the division did this peculiar univalent chromosome pass to one pole of the spindle (Figs. 17–22). In the metaphase plates of the second maturation division

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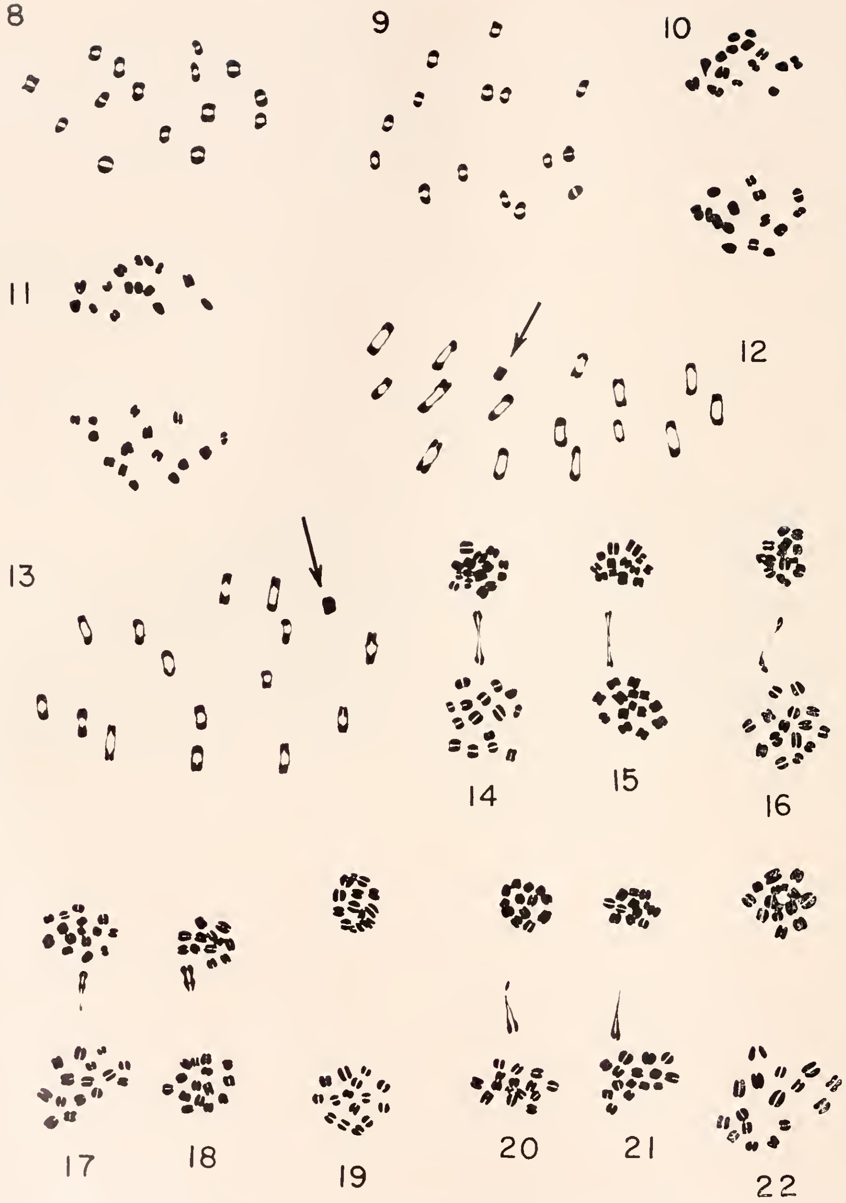
[3] Thirty to forty chromosome counts have been made for each of the represented stages.



Figures 8–22. Chromosomes of the large and the small eggs. Magnification: × 1500. Camera-lucida drawings.

Figures 8–9. Chromosomes of the first maturation division metaphase of the large male-producing egg.

Figures 10–11. Chromosomes of the first maturation division anaphase of the large male-producing egg.

Figures 12–13. Chromosomes of the first maturation division metaphase of the small female-producing egg. A univalent chromosome (indicated by an arrow) is found beside 15 bivalent chromosomes.

15 and 16 univalent chromosomes were counted. No lagging chromosomes were detected in the course of second maturation division.

5. *On a few exceptional females*

Of 25 parasitized crabs that the author reared, two were found to bear unusual females which produced both large and small larvae. The data concerning these females are here presented.

(*a*) *Five females on a host-crab* (♀) *of 12 mm. carapace length.* All of these females measured from 8.5 to 9.5 mm. in length of the external sac-like portion. One of these emitted a batch of nauplius larvae on October 10. When these nauplii were measured immediately after hatching, 90% of them had a body length of 225 to 240 μ. The remaining 10% of the larvae, on the other hand, measured from 250 to 260 μ in length. Thus, the nauplii emitted from this female consisted of many small larvae and a small number of large larvae. When these larvae were reared, small nauplii metamorphosed into typical small cypris larvae (240–270 μ) and large nauplii developed into large cypris larvae (280–295 μ). The four other females on the same host emitted numerous nauplii on October 10 and 11. In this case, each female also emitted many small larvae and relatively few large forms. The results of experiments showed that small larvae were destined to become females. Unfortunately, it was not ascertained whether the large larvae were functional male individuals, owing to the small number of the larvae.

(*b*) *Two females on a host-crab* (♂) *of 13 mm. carapace length.* There were 7 females on this host-crab. They measured 7 to 8 mm. in length. Five of them produced exclusively large nauplii which developed into large cypris larvae. The other two females produced both large and small nauplii which developed into large and small cypris larvae, respectively. In this case, the larger larvae were more numerous than the smaller ones.

The chromosomal constitution of the exceptional females described above was examined. It was found that the females which produced many small and few large larvae contained in their ovary oöcytes with 15 bivalent chromosomes and a univalent chromosome (the first maturation division metaphase was examined), while the females that produced many large and few small larvae had oöcytes with 15 bivalent chromosomes. Thus, the exceptional females in question were typical female-producing and male-producing females, so far as their chromosomal constitutions are concerned. Why did these females produce both large and small larvae? Although some tentative explanations might be presented here, the author would like to dismiss them until incontestable evidence is obtained.

REMARKS

According to Veillet (1943, 1945), the cypris larvae of *Triangulus galatheae* fall into two groups, the large cypris and the small cypris larvae. Although lacking direct evidence, Veillet maintained that a large cypris develops into an adult

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FIGURES 14–16. Chromosomes of the first maturation division anaphase of the small female-producing egg. A lengthened univalent chromosome is in the middle of the anaphase spindle.

FIGURES 17–22. Late anaphase of the first maturation division of the small female-producing egg. The univalent chromosome passes to polar body (17–19) or to egg nucleus (20–22).

hermaphrodite, while the smaller one functions as a larval male. In *Sacculina carcini,* according to Veillet, there is no larval dimorphism, but he conjectured that there might be two physiologically different kinds of cypris larvae, one a prospective adult hermaphrodite and the other the larval male. In *Peltogasterella gracilis*, as has been elucidated by the present study, there occurs a marked larval dimorphism. In this species, the large cypris represents the male sex and the small one represents the female sex. A notable fact is that an adult female rarely produces both male and female larvae. It produces regularly either male larvae or female larvae. In *Peltogasterella sulcata* (syn. *Gemmosaccus sulcatus; cf.* Reischman, 1959), Veillet (1952) observed that the cypris larvae derived from adult parasites on a host-crab (*Pagurus cuanensis*) successfully fixed to the crab, while those which were derived from the parasites on two other host-crabs (*P. cuanensis*) failed to fix upon the crab. Veillet made no mention of the reason for such facts. The present author surmises that the cypris larvae derived from the parasites on the one host-crab were female larvae and those from the parasites on the other two crabs were male larvae. The male larvae do not fix to the host-crab.

As examples of animals which are known to have the male-producing and the female-producing females in their population, the aphids (*Aphis* and *Phylloxera*) and the gall-fly (*Neuroteus*) may be mentioned (Morgan, 1912, 1915; Doncaster, 1916). In *Phylloxera caryaecaulis*, Morgan (1912, 1915) found that the eggs which are laid by the female-producing females, *viz.*, the female-producing eggs, are larger than those which are laid by the male-producing female, *viz.*, the male-producing eggs. Morgan observed that during the maturation division of the small male-producing egg two X-chromosomes lag behind the others in the spindle during anaphase, fail to enter the egg nucleus, and pass to the polar body. In the large female-producing egg no elimination of the X-chromosomes occurs. The fate of the egg (whether it develops into a female or into a male) is determined long before maturation division occurs. Why the oöcytes with the same chromosomal constitution develop into two different types of eggs has not yet been settled, although a hypothesis was formulated by Morgan (1915).

As another example of the animal which lays two morphologically and sexually different kinds of eggs, mention may be made of *Dinophilus apatris* (Archiannelida). Here, a female produces both large and small eggs which develop into females and males, respectively. The eggs are laid in a cocoon. The ratio of the large female-producing eggs to the small male-producing eggs is not always 1 : 1, but it varies with females and under influence of external conditions (Korschelt, 1882; Nachtsheim, 1919; Tzonis, 1938). The reason why the same female produces two different kinds of eggs is not yet fully understood. No cytologically distinguishable sex-chromosome has been demonstrated. In *Peltogasterella gracilis* there exists a special chromosome which behaves like a sex-chromosome. The female-producing female possesses this chromosome, while the male-producing female does not. The exact nature and function of this chromosome is unknown, and must be the subject of future research.

The author wishes to express his sincere gratitude to Prof. Atsuhiko Ichikawa (Hokkaido University) for his guidance and criticism during the course of this study. The author also wishes to thank Prof. Tohru Uchida and Prof. Sajiro Makino (Hokkaido University) for their interest and encouragement. Thanks are due to Prof. Emil Witschi (State University of Iowa) for his kind suggestions. To Dr. Placidus G. Reischman (St. Martin's College) the author is deeply indebted for reading the original manuscript.

Summary

1. The adult females of the rhizocephalan, *Peltogasterella gracilis* (Boschma), although they appear similar, are in reality two kinds, large-egg-producing and small-egg-producing females.

2. The large eggs develop into large larvae which function as males (larval cypris males). The small eggs develop into small larvae which give rise to adult females.

3. In the metaphase plate of the first maturation division of the large male-producing egg 15 bivalent chromosomes are seen, while in the small female-producing egg a univalent chromosome is found beside 15 bivalent chromosomes.

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ON THE ORIENTATION OF CENTRIOLES IN DIVIDING CELLS, AND ITS SIGNIFICANCE: A NEW CONTRIBUTION TO SPINDLE MECHANICS[1]

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The centriole is one of the most fascinating and baffling of the cell components. Despite the painstaking and inspired work done on this cell organelle during the last quarter of the nineteenth century, its very existence as a real structure was called into question within recent times (Fry, 1929). The idea that the centriole is a fixation artifact, without organic continuity, was refuted brilliantly by E. B. Wilson and his students, and the continuity of the centriole from one cell generation to another is now firmly established, for some materials at least (Wilson, 1930; Sturdivant, 1931; Wilson and Huettner, 1931; Johnson, 1931; Huettner, 1933). The observations on the centriole recorded by the older generation of cytologists, including Flemming, Boveri, Conklin, Lillie, Mathews, van Beneden, Heidenhain, Mead, MacFarland, and many others, were not only substantiated but actually reinforced by this controversy.

Despite all this interest, the actual function of the centriole in fertilization and cell division has remained essentially unelucidated, because of the anomalous situations existing in the *de novo* origin of centrioles during parthenogenesis, in the existence of primary and secondary centers during the normal fertilization of certain eggs, and in the well demonstrated existence of anastral and acentriolar mitosis in many forms, including the higher plants.

The great variation in form and behavior shown by this cell component, and by the centrosome, aster, blepharoplast and other structures related to it, is such, also, as to render controversial many of the hypotheses thus far made about the specific functions of the whole centriole-complex. The centriole itself may occur, in different materials, as a tiny dot-like structure; as a diffuse group of fine particles; as a definitive rod; as a distinct V-shaped body, with or without axial filaments and terminal vesicles; as a ring-shaped circlet; or there may be a complete absence of visible centrioles in certain anastral configurations. These variations present an array of manifestations which are, to say the least, confusing.

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Recent electron microscope studies (on all too few materials, thus far) have revealed, however, that the centrioles examined to date by this means are cylindrical cage-like bodies composed of 9 rod-like or tubule-like structures embedded in a matrix. The tubules may be double or single. Presumably the dot-like centrioles as seen by visible microscopy are actually very short cylinders, the longer rods longer cylinders, and the V's composed of two attached cylinders. The rings may be very short, very broad cylinders. No electron microscope studies have been made, to my knowledge, of the more specialized types of centrioles or of the finely particulate centriolar areas.

If the majority of centrioles are actually rod-like structures, however, it seemed that it might be profitable to examine one of the few forms in which the rod-shaped centrioles are sufficiently large to be easily distinguishable in fixed material without the use of electron microscopy. One of the purposes of the present paper is to give a more complete description of the remarkable giant first cleavage spindle of the egg of *Polychoerus carmelensis* and its unusual centrioles. Certain comparisons will be made, also, between the resting first cleavage spindle and the non-resting maturation spindles and their centrioles, but a more complete description of the meiotic spindles and their chromosomes will be reserved for a later paper.

Materials and Methods

Polychoerus carmelensis is a small orange-colored marine acoel flatworm from the tidepools of Pescadero Point, Carmel Bay, California, described by Costello and Costello (1938a). It is similar in many respects to *Polychoerus caudatus,* named and described by E. L. Mark (1892) and once common from Great Egg Harbor, New Jersey, to Casco Bay, Maine. Representatives of this genus are fascinating little animals, which as early as 1865 attracted the attention of A. E. Verrill (see his 1892–1893 paper), of Joseph Leidy in 1874 (see Verrill, 1892–1893), of T. H. Morgan in 1890 (personal communication from Ross G. Harrison), and later of E. G. Gardiner (1895, 1898). They have several unique or unusual features, certain of which have been mentioned earlier, in papers (Costello and Costello, 1938b, 1939) or brief abstracts (Costello, 1937, 1946, 1960a, 1960b, 1960c).

Since the egg of *Polychoerus* contains quantities of several types of orange pigment granules, yolk spheres, lipoid droplets, and nuclear remnants of incorporated vitellogenic cells, it is far too opaque for study of the living spindle. Only the dumbbell-shaped outline of the large amphiastral figure can be made out in living material. The primary oöcytes are fertilized and undergo maturation within the body of the hermaphroditic worm, after the worms have reciprocally mated, and there advance to the metaphase of the first cleavage division, at which stage they remain until the eggs are laid and enter sea water. Hence, an ample supply of fixed material, at stages up to and including the resting metaphase, can be obtained by examining the living adults with a hand-lens, and fixing those containing large oöcytes or ova visible through the body wall. The adult worms are quite hardy, and live well in a laboratory aquarium with running sea water, crawling about on the glass sides or wooden walls or on *Ulva* placed in the tank. They may also be kept in fingerbowls if the water is changed at intervals.

A variety of fixatives was used, including Heath's polyclad fixative, Worcester's, Boveri's picro-acetic, Flemming's, Lillie's modification of Meves' fluid, B-15, 20%

formalin, Champy's, Carnoy and Lebrun's, Regaud's #1, and Gilson's fluids. The majority of these were used at three different temperatures: room temperature, warm (about 50° C.) and hot (60–80° C.), with specific temperature records kept in each case. Some egg masses were fixed, also, at various stages after having been laid, and 12 adults were fixed in the act of egg-laying (see Costello and Costello, 1939). All this material was collected and fixed during the author's stay at the Hopkins Marine Station, Pacific Grove, California, during the summers of 1936 and 1937. I am indebted to the late Director, Dr. W. K. Fisher, and his staff for many courtesies.

The fixed animals were individually embedded in hard paraffin, and sectioned serially at 8 or 10 microns. Approximately equal numbers were sectioned transversely, frontally and sagittally. The serial sections were stained by a variety of methods: Heidenhain's iron haematoxylin, Feulgen, Champy-Kull, Benda, Flemming tricolor, safranin, Delafield's haematoxylin, Mallory's triple stain, anilin blue, or crystal violet, and counterstained, in some cases, with eosin, erythrosin, light green, or orange G. Specimens fixed in room temperature Worcester's solution or in Heath's solution and stained with Heidenhain's iron haematoxylin gave preparations of superlative beauty. More than 423 sets of serial sections of the worms and egg masses were prepared by my wife and myself in 1936, 1937 and 1938. Most of these slides were studied by Helen M. Costello and the writer between 1936 and 1940. All the preparations were subsequently re-studied by Dr. Catherine Henley in 1958. This paper would not have been written without this help, and I hereby acknowledge my deep appreciation. My thanks are due, also, to Drs. Sally Hughes-Schrader and Franz Schrader for many stimulating discussions about the *Polychoerus* egg over a period of years, and for reading the manuscript; to Dr. Kenneth W. Cooper for similar conferences; and to Dr. Shinya Inoué for an all-too-brief discussion at Woods Hole during the summer of 1960.

The serial sections were examined at various magnifications (50 to 2400 diameters) and the location (slide number, row number, section number) of each significant feature noted, to serve as a record and in order that any feature might easily be found again. Photomicrographs were taken of maturing eggs, spindles, chromosomes, etc., at magnifications of 300 to 1400 diameters, using apochromatic objectives and compensating eye-pieces. Wash drawings were made by Mrs. Ernest Runyon, to whom I am greatly indebted.

Observations

The giant cleavage spindle of the egg of Polychoerus

The fully grown eggs of *Polychoerus carmelensis*, after fixation, measure 200 to 280 microns in diameter. Considerable shrinkage in certain of the fixatives is indicated by the fact that the cavities in the parenchyma in which the eggs lie are 260 to 320 microns in diameter. In one animal fixed in Champy's fluid, there were no shrinkage spaces around the eggs, and one ovum, just extruded through the body wall, measured 320 microns in diameter. There is every reason to believe that the ova are closely surrounded by the parenchyma when the animals are alive, and that the eggs shrink more than the surrounding tissues.

The resting (metaphase) first cleavage spindle of *Polychoerus* is of the amphiastral type, with the continuous fibers constituting a central spindle (Hermann,

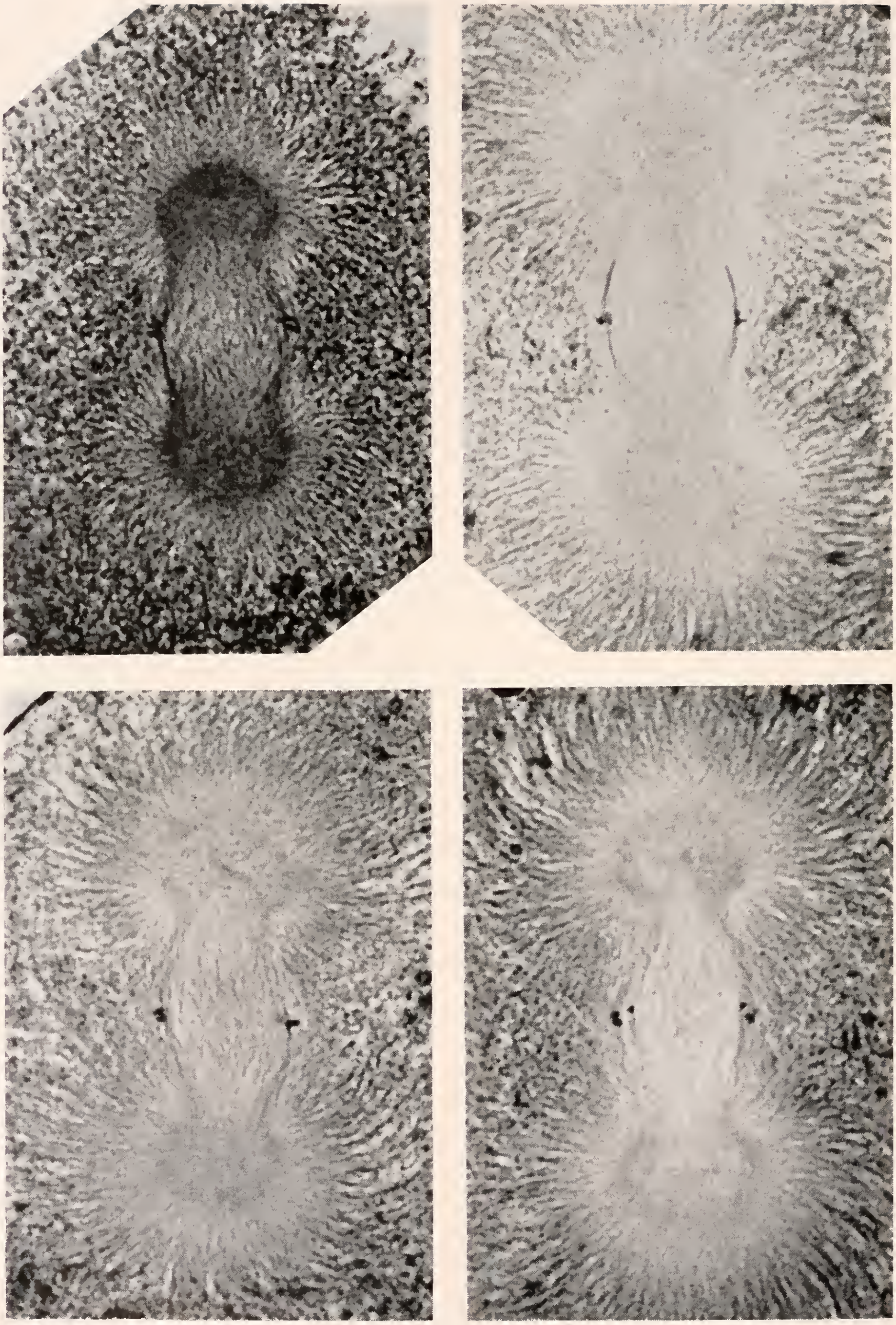

FIGURE 1. Photomicrographs of 10-micron sagittal sections through resting first cleavage metaphase of four eggs of *Polychoerus carmelensis.* Left, upper, fixed in Flemming's; right,

1891) about which the chromosomes are grouped in a ring (Fig. 1). A sheath of mantle fibers lies peripheral to the central spindle, with the chromosomal fibers attached to the kinetochores of the fringing chromosomes. The chromosomal fibers are more fibrous and less granular than the continuous fibers or the astral rays. The chromosomal fibers appear, also, to be compound, consisting of as many as 10 or 12 finer fibrils. The chromosomes are all V- or J-shaped, with median or sub-terminal attachments, and with their arms projecting radially outward from the equatorial plate (Fig. 13). The diploid number of chromosomes, repeatedly counted with ease in a large number of the resting metaphases, is 34. The chromosomes are not exceptionally large, the long arms of the J-shaped chromosomes being 5 or 6 microns in length and the arms of the V-shaped a little less. These chromosomes in the resting metaphase are never double, and have not yet visibly split at this stage.

The fixed spindle at resting metaphase measures up to 120 microns in length[2] between the centers, and a maximum of 65 microns in diameter at the equator. The spindle plus asters often exceeds 160 microns in length. The usual diameter across the equatorial plate is 45 to 50 microns. It should be noted that these dimensions are those of sectioned material; therefore, they do not give the distorted and exaggeratedly spread relationships so often seen in squashed preparations.

In many preparations (especially those fixed in Heath's and heavily stained in iron haematoxylin), a distinct centriole can be seen at each astral center, in most cases in the shape of a rod which may sometimes be straight, but is more often slightly curved, and in a few cases, somewhat twisted. In other eggs, fixed less well, the central region of the aster may show a fine granulation, but no distinct centriole. When curved, the centrioles have their free ends nearer the equatorial plate, the convex portion away from it.

The rod-shaped centrioles of the cleavage metaphase of *Polychoerus* are 4.5 to 5 microns in length by about 0.25 micron in diameter. Examination of large numbers of cleavage spindles sectioned longitudinally indicates that the centrioles at the two ends of a given spindle are never oriented parallel to each other. In fact, while they lie transversely (or, more rarely, slightly obliquely) to the long axis of the spindle, their axes never lie in the same plane but are oriented at right angles to each other and usually at right angles to the spindle axis. Under intermediate magnification, this often results in a figure that gives the superficial appearance of having a rod-shaped centriole at one pole and a small spherical centriole at the other (see Figure 2). Under high magnification (of the light microscope), the spherical centriole can be seen to be the optical section of a vertical rod, at right angles to the horizontal rod-shaped centriole at the other pole. In eggs in which the plane of section did not happen to coincide with the longitudinal axis of the spindle, the same right-angle relationship between the two centrioles of a given spindle can be demonstrated by studying the serial sections. Whatever the angle of orientation of one centriole in relation to the plane of section, the centriole at the

[2] In the ovum fixed in Champy's fluid, the spindle measured 134 microns between the centers and 40 microns in diameter at the equatorial plate. The asters were about 80 microns in diameter.

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upper, and both lower fixed in hot Heath's; all stained in Heidenhain's iron haematoxylin. The slender rod-shaped centrioles (0.25 micron in diameter) do not show clearly at this magnification. 550 ×.

other end of the same spindle was found to be approximately at right angles to the first.

Electron microscope studies of centrioles by Burgos and Fawcett (1955), by Porter (1956) and by de Harven and Bernhard (1956) indicate that centrioles are cylinders, consisting of 9 rods in a matrix, as mentioned above. In repeated cases in vertebrate cells, where two centrioles are found close together and therefore probably recently divided, the bundles of rods are oriented at 90° to each other. Here, in this resting spindle of *Polychoerus*, even though the centrioles are at opposite poles, they are also oriented at 90° to each other. Since the centrioles of most vertebrate cells are tiny, measuring about 0.3 to 0.5 micron in length by 0.15 micron in diameter, there is not much chance of discovering the orientation of both of them in a given spindle by light microscopy in any appreciable number of cases. The light microscope does not give a high enough magnification to reveal their orientation. The thin sections used in electron microscopy are unlikely to include both centrioles of a spindle. However, de Robertis, Nowinski and Saez (1960) figure a thin-section electron micrograph (by Bernhard) which includes both poles of a cell in metaphase. Two centrioles are present at each pole, with the recently divided daughter centrioles at each pole at right angles to each other. The distal centriole of each pair is sectioned longitudinally, the proximal centriole transversely, in this particular case. This spindle, however, measured only 5.5 microns (calculated from the stated magnification) between the proximal centers.

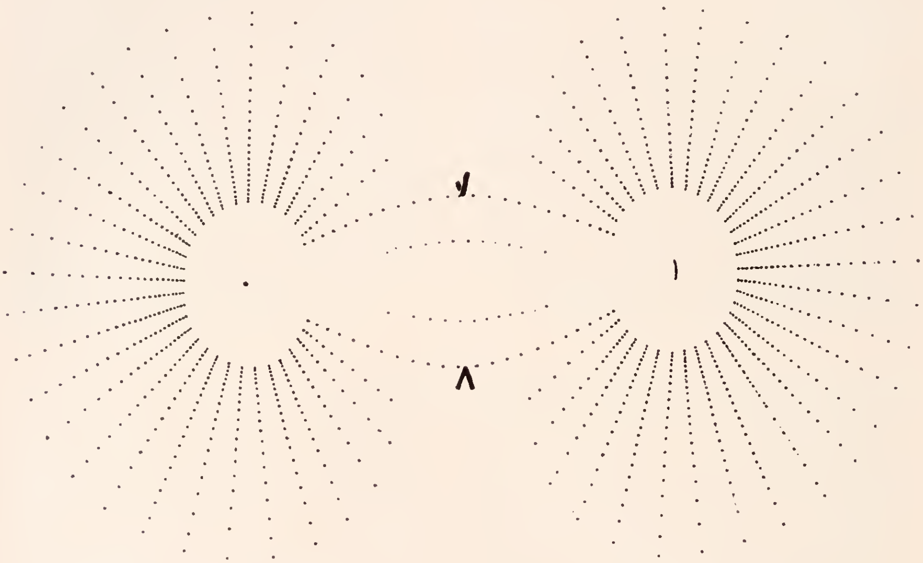


FIGURE 2. Diagram of resting first cleavage metaphase spindle of *Polychoerus*, to show centriolar orientation.

Cells of the size of *Polychoerus* eggs are rare and giant spindles are practically unknown throughout the animal kingdom. So far as I have been able to ascertain from the literature, only the spindles of the cleaving egg of the whitefish approach those of *Polychoerus* in size. In the whitefish, chromosomes extend throughout the equatorial plate, and there is no distinctive central spindle. Consequently, the details of spindle structure and chromosomal arrangement are far less clear.

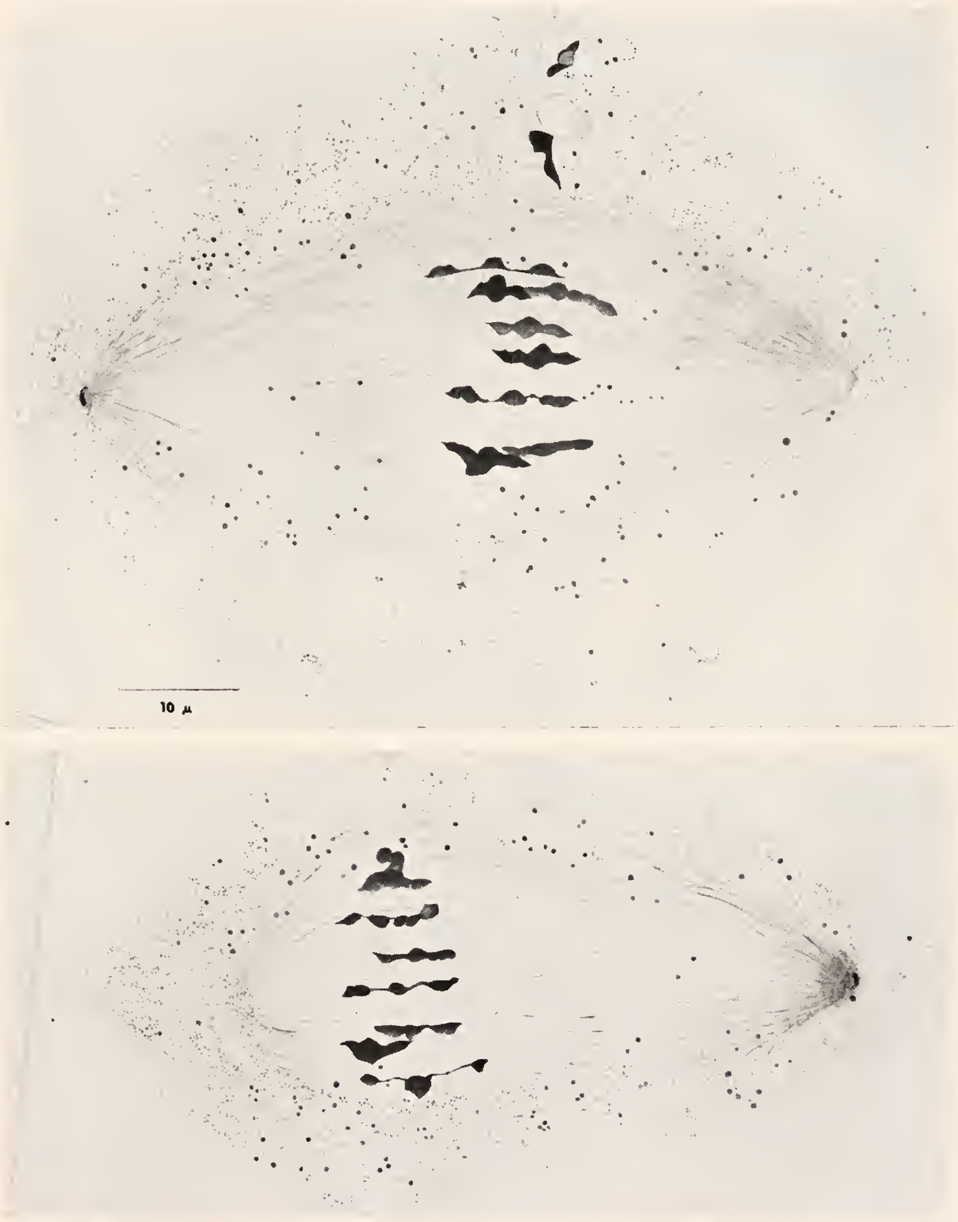


FIGURE 3. Wash drawings, of two adjacent 10-micron sections through metaphase of first maturation spindle of primary oöcyte of *Polychoerus*. Fixation in Worcester's at 60°, stained with Heidenhain's iron haematoxylin and eosin. Figures 3, 4 and 5 were drawn by Laliah Curry Runyon (Mrs. Ernest Runyon).

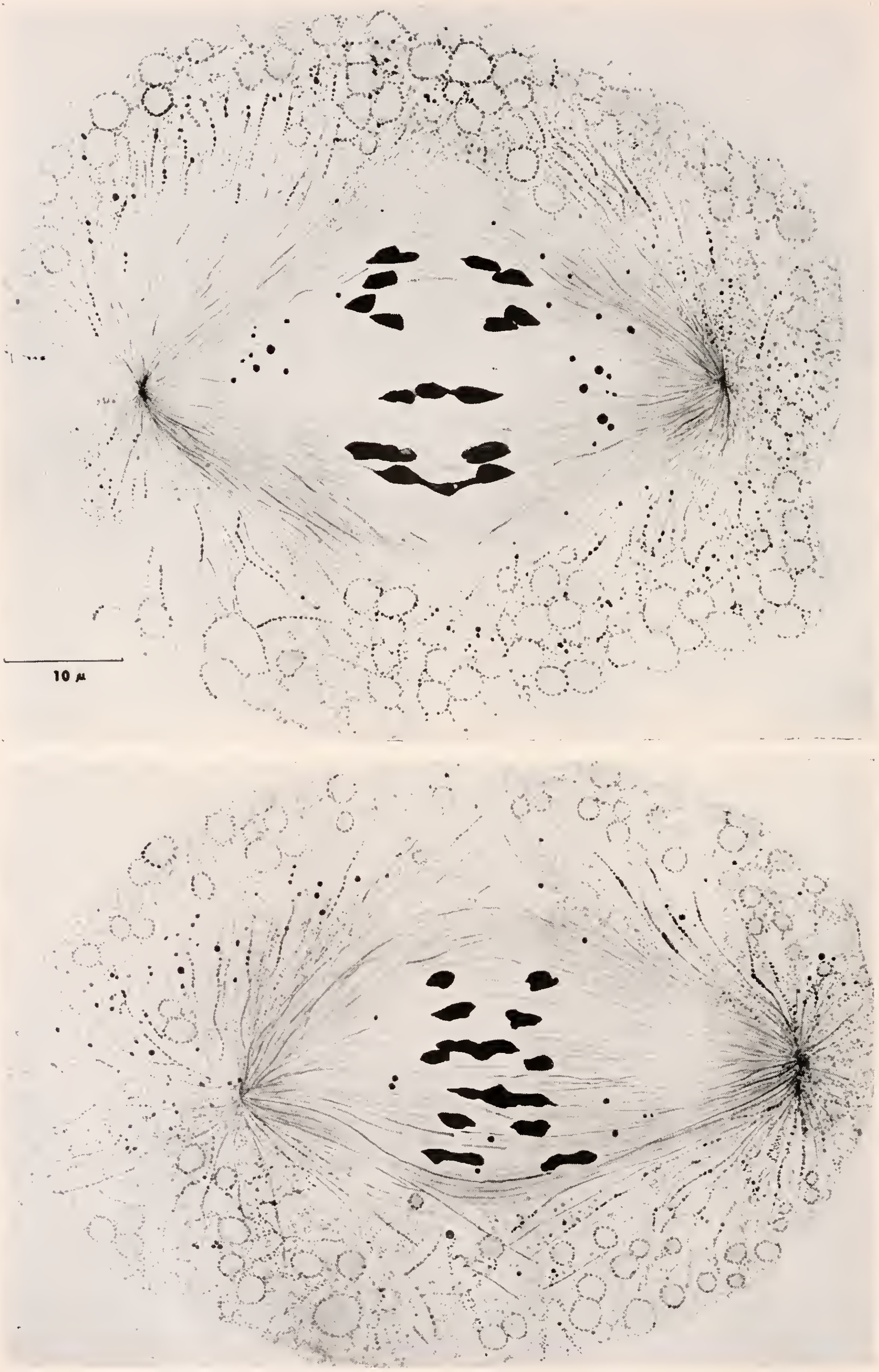


FIGURE 4. Wash drawings of two adjacent sections through first maturation anaphase of primary oöcyte of *Polychoerus.* Fixation: Heath's at room temperature; staining, Heidenhain's iron haematoxylin.

When one considers that one of the most favorable (and largest) cells used for the study of the mitotic spindle, namely, the grasshopper neuroblast, is only 30 microns in diameter, and that most mammalian cells are much smaller than this, the advantages of a form like the *Polychoerus* egg are apparent.

Giant centrioles are, however, not unknown. Cleveland (1935a, 1935b, 1935c, 1938, 1957a, 1957b) has described centrioles (in members of the flagellate genera *Pseudotrichonympha* and *Barbulanympha*) up to 80 microns in length, and in these and in other flagellates, such as *Spirotrichonympha* (Cleveland, 1958), the demonstrated relations between these and the central spindle and chromosomes are rapidly advancing our knowledge of the functions of the centrioles.

The centrioles of the first maturation spindle of the egg of Polychoerus

Since the sectioned material included many animals containing oöcytes fixed while undergoing the maturation divisions, it was imperative to examine these, also, to ascertain the structure and orientation of the centrioles, for comparison with those of the first cleavage spindle. The first maturation spindle of *Polychoerus carmelensis* measures about 75 microns between the centers at metaphase. It is 25 microns in diameter at the equator. Instead of having a central spindle consisting entirely of continuous fibers, there are at least three chromosomes located within the spindle, while the others are more peripherally arranged. This has been observed in transverse sections through the equatorial plate region. The asters of the first maturation division are much less well developed than those of the cleavage spindle, but in the better-fixed material, centrioles are clearly visible at each pole. These centrioles are relatively small, measuring about 1 micron in length by about 0.25 micron in diameter. Some variations in form of the centrioles are found at various stages during the first meiotic division. At the pre-metaphase stretch stage and also at metaphase, centrioles have been found in the shape of curved rods, which may or may not have slightly enlarged ends. These curved rods are oriented with the convex side away from the equatorial plate, the concave side (and free ends) extending toward it. An imaginary line across the free ends of the centriole at one pole is approximately at right angles to the long axis of the spindle, and in almost every case examined was found to be *at right angles to the centriole axis* at the opposite pole of the same spindle. In other spindles at approximately the same or a later metaphase stage, the centrioles were distinctly double at each end of the spindle, consisting of two short rods (0.75 to 1 micron by 0.25 micron), end-to-end or at a slight angle to each other. The apex of the angle was always directed away from the equatorial plate. Obviously these are bivalent centrioles, and they are also oriented at right angles to each other at the opposite poles of each spindle (see Figure 4). There is very little distance (0.1 to 0.2 micron) between the apices of the two rods, and it is possible that some of the centrioles which looked like single curved bodies actually consisted of two parts. They may, however, represent stages immediately preceding duplication of the centrioles, but this requires further detailed study.

There are 17 tetrads on these first meiotic spindles of *Polychoerus carmelensis*. A description of them, and of the stages of breakdown of the germinal vesicle, will be the subject of a later paper. Meanwhile, it may be mentioned that the first meiotic spindle originates as a central spindle of *extranuclear origin*, generated as

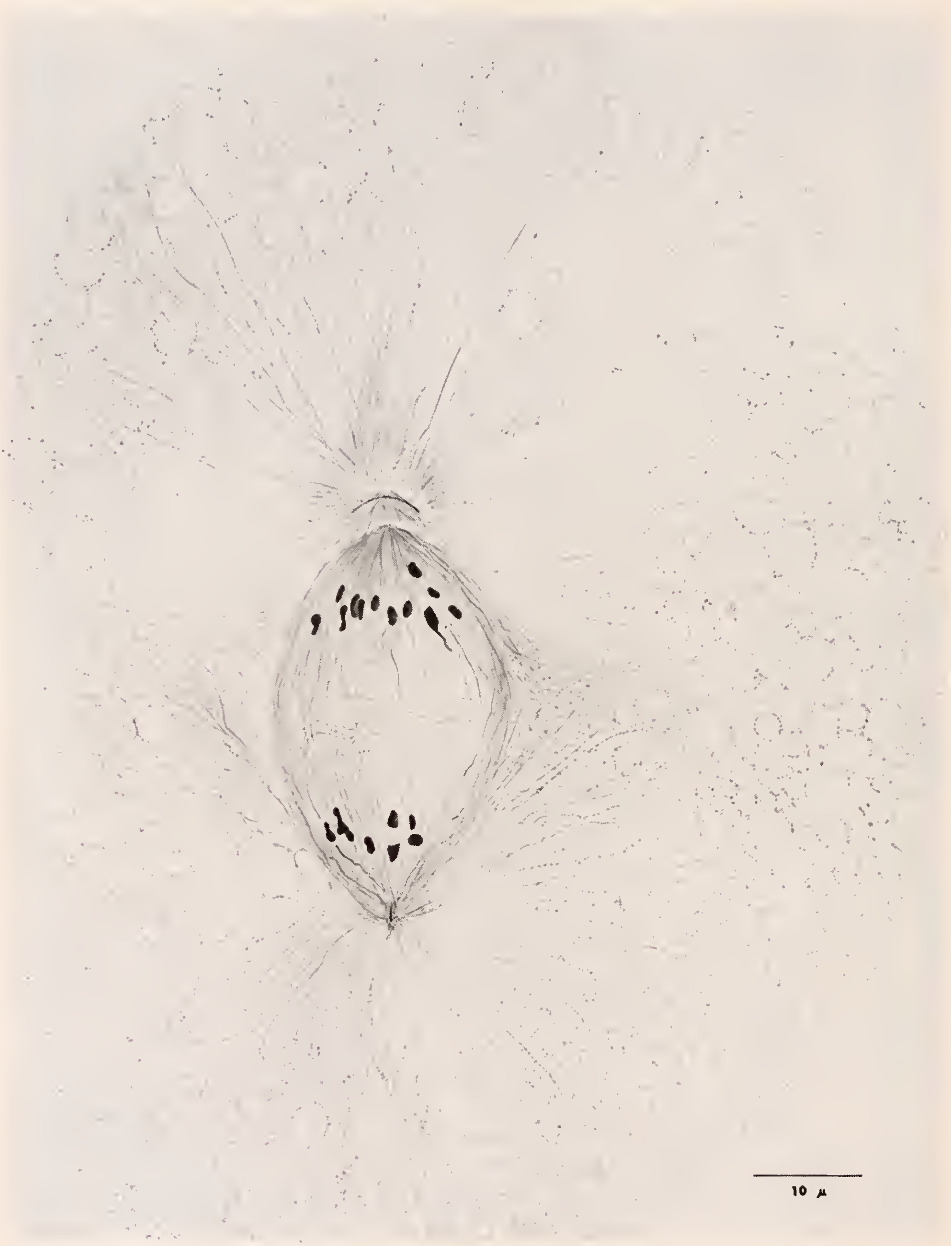


FIGURE 5. Wash drawing of 10-micron section through second maturation anaphase of a secondary oöcyte of *Polychoerus*. Fixation in Worcester's at room temperature; stained in Heidenhain's iron haematoxylin and eosin.

the two primary centrioles and asters move apart and around the oöcyte nucleus, before the wall of the germinal vesicle shows any signs of rupturing. At the same time, in numerous primary oöcytes, smaller secondary centers (with asters), between 5 and 20 in number, appear in the cytoplasm near the wall of the germinal vesicle. These secondary centers may even persist at various points in the cytoplasm, long after the first maturation spindle with its primary centers is fully established. The secondary centers are too tiny for an analysis of their shape. After the rupture of the oöcyte nucleus, the chromosomes become attached to the central spindle and undergo marked pre-metaphase stretch movements.

The centrioles of the second maturation spindle of the egg of Polychoerus

There were not many second meiotic spindles found. These are relatively shorter, broader spindles (38 microns long by 21 microns in diameter at anaphase) than those of the first meiotic division, and contain 17 dyads at metaphase. The astral regions are somewhat better developed than in the first meiotic spindle. The centrioles of these second meiotic spindles are truly remarkable. They are long, thin bodies, curved into a crescentic form. The clearest of these are 5 microns in length and so thin as to be just at the limit of microscopic visibility (*i.e.*, about 0.2 micron). The concave side (and free ends) are directed toward the equatorial plate, the convex side away from it. These resemble the centrioles of the second spermatocyte division of *Nemobius,* as described (his Fig. 39) by Baumgartner (1929), although in the gryllid material the centrioles are straight. At the two opposite ends of the same spindle, the axes of the centrioles are again at right angles to each other (see Fig. 5). There is no indication of duplication in any of these second meiotic centrioles. These appear, then, to be univalent. Presumably because of the orientation of the two centrioles, these second meiotic spindles look much more heteropolar than did the first.

Since the metaphase centrioles at the opposite poles of the first maturation spindle, of the second maturation spindle, and of the first cleavage spindle, even though differing in size, valency, etc., are oriented at right angles to each other, it would seem that this is an inherent property of either the centrioles or the protoplasm of the species.

The details of the origin of the cleavage centers for *Polychoerus carmelensis* have not yet been completely worked out. However, enough stages have been studied (including many examples of the association of the 17 maternal with the 17 paternal chromosome vesicles) to indicate that the process in *Polychoerus* is probably not unlike that in most other forms; thus far, however, a sperm nucleus has not been seen at the stage when it bears a sperm aster. Gardiner (1898) figures such a stage (his Fig. 24) for *Polychoerus caudatus.* At the period of association of the maternal and paternal chromosome vesicles, two rod-shaped centrioles, with well developed asters, are frequently found (Fig. 6). Occasionally the two centrioles and asters are sufficiently far apart as to appear to be associated with separate groups of chromosome vesicles. These centrioles resemble those of the first cleavage metaphases in size and shape. The astral rays can be seen to originate along their entire lengths.

Curiously enough, I have thus far been unable to identify with certainty any centrioles (or asters) in the first or second meiotic divisions of the spermatocytes, which are found in other regions in the same serial sections of the same (hermaphroditic) animals that show such beautiful egg centrioles and asters. Fusiform spindles with tetrads or dyads are clearly seen, and the material appears to be excellent for a comparison of sperm chromosomes with egg chromosomes at corresponding meiotic stages. Large, distinct black dots (after haematoxylin staining) in the spermatocytes are undoubtedly the chromatoid bodies.

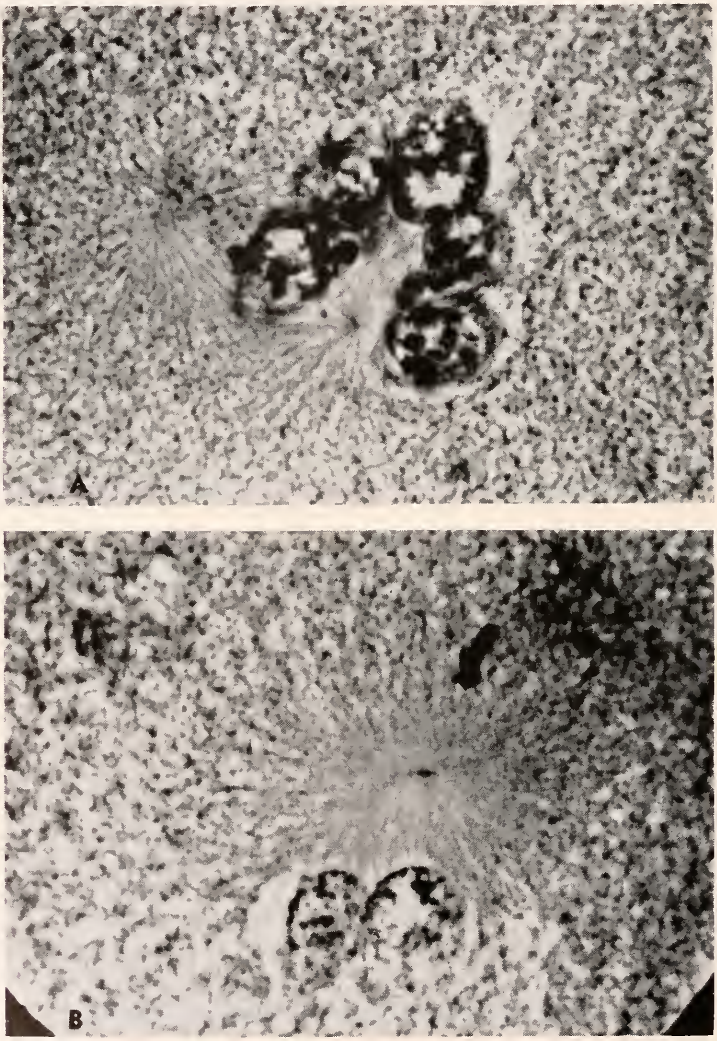


FIGURE 6, A. Beginning of cleavage diaster, and 6 of the 34 chromosome vesicles. The centriole between the vesicles is a rod, in plane of section. The other centriole is perpendicular to plane of section, and half is in the adjacent section. 910 ×.

FIGURE 6, B. Aster and one rod centriole of early cleavage diaster, associated with two chromosome vesicles, in another egg. Centriole foreshortened. Fixation: hot Heath's; stain: Heidenhain's iron haematoxylin. 910 ×.

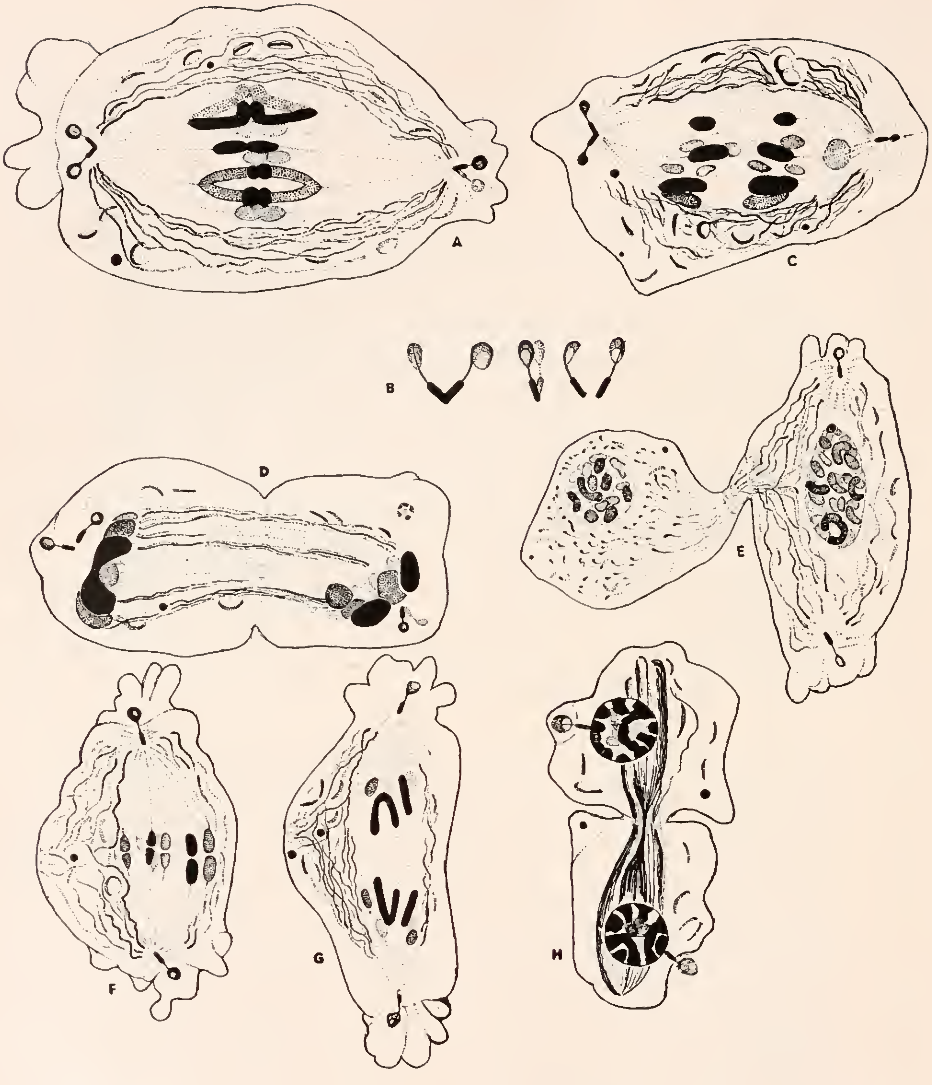
Discussion

Related observations made by others

The bivalent centrioles found in the first maturation division of *Polychoerus* are reminiscent of the V-shaped centrioles of the Gryllidae described in Johnson's excellent paper (1931), even though the V-shaped centrioles are oppositely directed as compared with those of *Polychoerus.* Johnson found that the centriole of the spermatogonium is a very short rod, scarcely longer than it is broad, frequently tilted at an angle to the long axis of the spindle. These centrioles are found at opposite ends of the nucleus *before* spindle formation. When the pachytene stage of the primary spermatocyte is reached, all the cells exhibit centrioles of a definite V-shape, directed apex inward, the tip of each limb in contact with the cell membrane. Johnson observed the migration and separation of these V-shaped centrioles in *living* material, and figured (his Figs. 83, A and B) the *rotation* of *one* of the two central bodies which had taken place over an interval of five minutes. He states (p. 124), "One central body nearly always lies in a plane at right angles to its fellow of the opposite pole, as seen in Fig. 35 [Figure 7,C of the present paper], in which the upper one is lying flat while the other is on edge. Rotation into this position is accomplished at various times during the prophase."

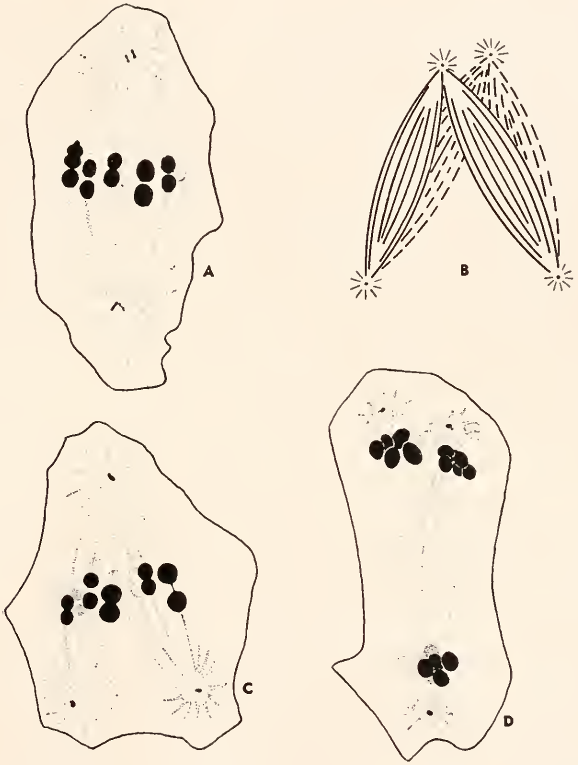
In *Oecanthus* and other Gryllidae studied, a split ocurs at the apex of the V at anaphase of the first meiotic division. This gives rise to centrioles of the second order which separate as short rods, each with its axial thread and terminal vesicle. These rods move apart 180° as the spindle for the next meiotic division is established. The second division spindle therefore forms at right angles to the first. However, Johnson (pp. 125–126) continues: "When the V-shaped central apparatus breaks into rods, the moieties move apart in that plane in which the arms previously diverged. Since the V's usually lie at right angles at the primary metaphase, the two secondary spindles will not only lie at right angles to the primary axis but also at right angles to each other. . . . That divisions of secondary cytes actually occur as I have indicated is quite evident in examination of second division cysts. If one cell is cut sagittally, its closest neighbor is usually cut transversely." (See Fig. 7,E in the present paper.) At metaphase of the second meiotic division the centriole rods of *Oecanthus* lie more or less directly in the longitudinal axis of the spindle, but sometimes, especially at anaphase, the elements are slightly tilted (Fig. 7,G in the present paper). Baumgartner (1929) described similar centriolar behavior during the spermatogenesis of another gryllid, *Nemobius fasciatus.*

Another equally interesting case is presented by Schrader (1941) in his paper on spermatogenesis in the earwig, *Anisolabis.* During the first meiotic division of the spermatocyte there are two centrioles visible at each spindle pole. He states (p. 136), "Each centriole is evidently a short rod. The axes of the two centrioles in each centrosome have no definite relation to each other and almost every possible variation has been encountered." Many of these cells divide normally (see Fig. 8,A of the present paper), while certain other cells exhibit a most interesting anomaly of spindle behavior (Figs. 8,C and D). As Schrader states (pp. 129–130), "In such cells the two centrioles at each pole, which normally remain closely associated in a single centrosome, begin to separate—usually just about the time that the metaphase is being established. . . . The precocious separation of the centrosomes occurs simultaneously at both poles of the cell involved. . . . The movement of



FIGURES 7, A through 7, H redrawn by Helen M. Costello from Johnson (1931), *Zeitschr. wiss. Zool.*, 140, with the permission of the publisher and author. All are of *Oecanthus nigricornis.* (A) First meiotic metaphase in primary spermatocyte. Centrioles at right angles to each other, with axial filaments and terminal vesicles. (B) Centrioles from first meiotic figures in front and side views. Daughter centrioles at right, after division of a V-shaped centriole. (C) First division, anaphase. The centriole at the right pole is in end view. (D) First division, telophase. Division and beginning migration of daughter centrioles. (E) Secondary spermatocytes at interphase. Adjacent daughter cells connected by spindle-bridge from the first meiotic division. Note the relation of the cell axes. The left cell is cut in transverse section. (F through H) Second meiotic division, metaphase, anaphase and telophase, respectively. Note the asymmetry of distribution of the chondrioconts in (F) and (G), and the telokinetic rotation of the nuclei and centrioles in (H). The dark dots are chromatoid bodies.

the centrosomes after separation is usually very definite. It is such that a plane passing through the original axis and the sister centrosomes at one pole is nearly always at right angles to one passing through the pair at the opposite pole. . . . The result is a quadripolar figure which, if it shows the centrosomes of one pole at the same focal level, will present the opposite pair of centrosomes optically superimposed on each other." (See Figs. 8,B and D of the present paper.)

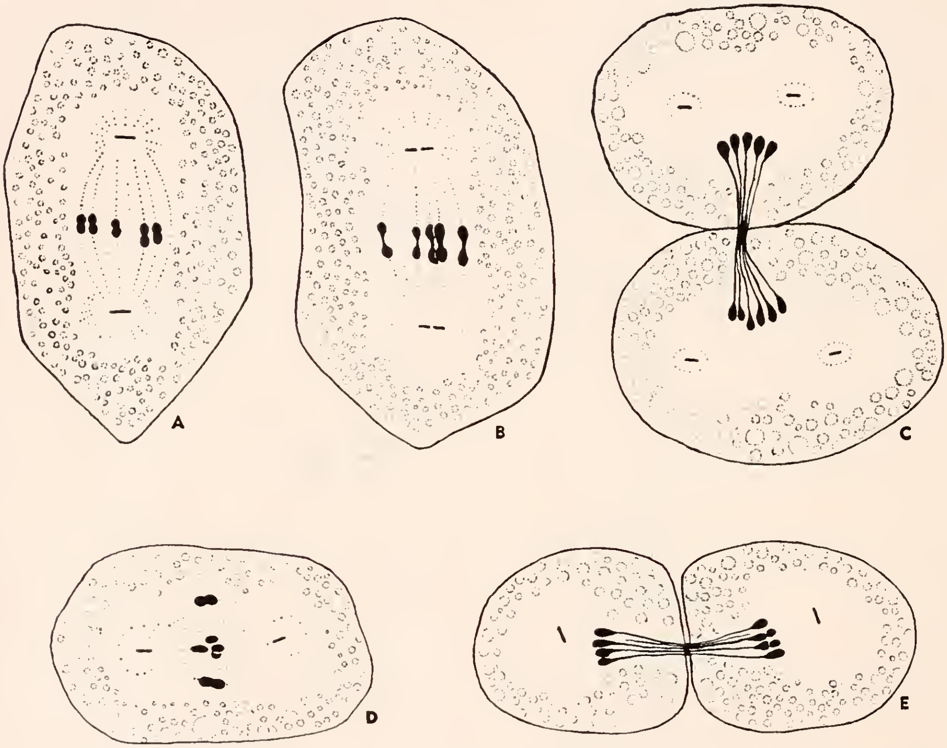


FIGURES 8, A through 8, D redrawn from Schrader (1941), *J. Morphol.*, **68**, with permission of the publishers and author. All are of *Anisolabis maritima.* (A) Normal first spermatocyte metaphase, in side view. (B) Diagram showing the four spindles that arise after precocious separation of the centrioles in two planes. (C) First meiotic metaphase with precociously divided centrosomes. Those at upper pole superimposed on each other and in a plane at right angles to those of lower pole. Only two of four spindles shown. (D) Telophase of first meiotic division, with precocious centrosomal division. The centrosomes at the lower pole superimposed on each other. Chromosomes in two groups at the lower pole.

The *Anisolabis* material of Schrader therefore shows a type of centriole behavior similar to that described by Johnson (1931) for *Oecanthus,* except that the *precocious separation* of the centrioles in the anomalous *Anisolabis* cells did not give rise (Schrader, p. 130) to the production of continuous fibers (*i.e.,* a spindle) between them.

In the spermatogenesis of the hemipteran *Gelastocoris,* as described by Payne

(1927), a different situation obtains. Payne states (p. 319), "[In the primary spermatocyte] the centriole is still single and a straight rod and lies at right angles to the long axis of the spindle [Figure 9,A in the present paper]. As the chromosomes constrict and begin to move apart the centriole divides transversely into two equal parts, which gradually separate as the chromosomes move toward the poles of the spindle [Figure 9,C in the present paper]. The second division follows without a nuclear reconstruction and the division figure is built around the two new centrioles which now lie more or less parallel to the long axis of the spindle [so that both spindles of daughter secondary spermatocytes lie in one flat plane]. As the chromosomes approach the poles in this division, the centriole moves to one side and lies here during the formation of the spermatid nucleus [Figure 9,E in the present paper]. . . ."



FIGURES 9, A through 9, E redrawn from Payne (1927), *J. Morphol.*, 43, with permission of publishers and author. All are of *Gelastocoris oculatus*. (A) Centriolar orientation at metaphase of the first spermatocyte division. (B) Early anaphase of the first spermatocyte division. The centrioles have divided. (C) Late anaphase of the first meiotic division. Centrioles have moved apart to become the centrioles for the second division. (D) Metaphase of second meiotic division. Centrioles have retained orientation in each daughter cell resulting from first division. (E) Telophase of the second meiotic division. Centrioles have turned and moved to one side in each daughter cell (spermatid).

Thus we have two strikingly contrasting conditions of cell orientation in

Oecanthus and *Gelastocoris*, associated with differences in *centriolar orientation* and *behavior*.

Since in both these cases we are dealing with terminal divisions in spermatogenesis, the further significance is not immediately apparent.

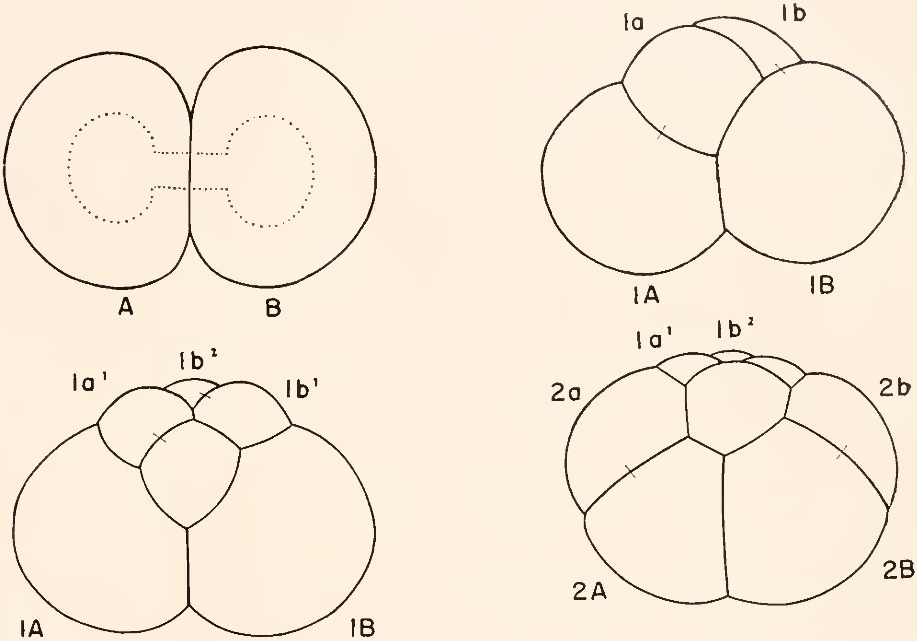


FIGURE 10. The early cleavages of *Polychoerus carmelensis* drawn from photomicrographs of living eggs. Egg membrane (chorion) and external jelly omitted. The cleavage of *Polychoerus carmelensis* differs in many details from that described by Bresslau (1909, 1933) for *Convoluta*, and by Gardiner (1895) for *Polychoerus caudatus*. (Upper left) Two-cell stage at the time of completion of the first cleavage furrow. The amphiaster is visible. (Upper right) Four-cell stage, side view. Two micromeres (1a, 1b) have been formed by a leiotropic second cleavage. (Lower left) Six-cell stage, side view, slightly tilted toward the observer. The members of the first duet of micromeres (1a, 1b) have divided by a dexiotropic cleavage, to give $1a^1$ and $1a^2$, and $1b^1$ and $1b^2$, and there has been some shifting of the blastomeres. (Lower right) Eight-cell stage, side view. The macromeres 1A and 1B have divided almost bilaterally but somewhat dexiotropically, producing 2a and 2b, and becoming 2A and 2B.

The possible significance of centriolar orientation

The *Polychoerus* material gives information not only on the orientation of centrioles during oögenesis, but also on their orientation for first cleavage. Omitting, for the moment, a discussion of the situation regarding the formation of the polar bodies, the possible significance of the relation of centriolar behavior to cleavage may be considered.

The first cleavage of the egg of *Polychoerus* is equal (or near-equal), giving rise to two cells, A and B (Fig. 10), with presumably identical cytoplasmic composition. The first cleavage plane bisects the large spindle and presumably passes through the animal pole, although this pole is not marked by any polar bodies. As I have pointed out briefly (Costello, 1960b), the polar bodies are not extruded but are intracellular, mark no axis, and eventually degenerate.

The right-angle orientation of the slightly curved centrioles to each other and to the spindle axis has obviously not influenced this first cleavage. The axes of the centrioles themselves, as also the single or duplicated state, bear no necessary relation to the spindle axis of that cleavage in which they are being observed. Evidence for this general conclusion is given, also, by the *Gelastocoris* centrioles, the axes of which are at right angles to the spindle axis in the first meiotic division and coincide with it for the second; by the *Oecanthus* centrioles, which are V's with apex toward the spindle at the first meiotic division and are rods roughly parallel to the spindle in the second; and by the *Polychoerus* centrioles which are slightly divergent paired rods (with apex away from the spindle) at the first maturation division and are longer curved rods at the second polar division.

However, it is immediately apparent what will happen at the second cleavage division of the *Polychoerus* egg if a similar pattern of inherent centriole behavior is followed. That is, the rod centrioles which are at right angles to each other in the first cleavage figure will each produce daughter centrioles and these will separate in a slightly oblique direction from each other, and at right angles in blastomere A as compared with blastomere B, generating the second cleavage spindle as they move apart. This oblique separation could be due to the slight curvature of the centriolar rods, especially if one daughter centriole of each blastomere were free to move more readily through the protoplasm toward (and to the left) of the animal pole, while the other might be unable to migrate as easily into the more densely yolk-packed vegetal region. It might also be related to the manner of origin (and hence orientation) of the daughter centrioles as they are producd. The members of the pairs of daughter centrioles in each blastomere will likewise be at right angles to each other, the upper one nearer the animal pole and at right angles to the lower one, near the middle of the vegetal hemisphere. The two oblique second cleavage spindles must likewise be assumed to be heteropolar, with the upper ends differing from the lower ends (like the heteropolar auxocyte of *Oecanthus* shown in Johnson's Fig. 5). This might be due to the type of protoplasm (animal versus vegetal) in which the ends lie. When this second cleavage is completed, we would then have, by a typical *leiotropic spiral cleavage,* a pair of micromeres, 1a and 1b, surmounting a pair of macromeres, 1A and 1B; this actually results at the second cleavage of the *Polychoerus* egg (Fig. 10, upper right).

If a similar pattern of centriole behavior is repeated at the third cleavage, with the formation of spindles determined by the axes of daughter centriole separations, the result would now be a *dexiotropic* cleavage for each cell that had arisen leiotropically. When this cleavage cycle is complete, we would have an 8-cell stage, with four micromere products ($1a^1$, $1a^2$, $1b^1$, $1b^2$), two second duet micromeres (2a, 2b) and two second generation macromeres (2A, 2B) (Fig. 10, lower right). We can thus explain the *regular alternation of leiotropic and dexiotropic* cleavages in the early development of this form, and, *presumably, in all other spirally cleaving forms.* (*Polychoerus* shows spiral cleavage by duets, practically all forms other than acoels show spiral cleavage by quartets. The mechanics of centriole behavior in the quartet type could be similar, but more complex.)

One can, therefore, set up the general hypothesis that the orientation of the centrioles with respect to each other and with respect to cell polarity, at any given division (meiotic, mitotic [or cleavage]) in which centrioles are normally present, determines the *original* position in which the daughter centrioles will separate from each other. This, in turn (provided there are not secondary re-orienting factors operating), *determines the main axis of the spindle for the next division, and hence* (barring the intervention of secondary factors), *the relative orientation of cells to each other* in the embryo, organ, or even organism. This orientation of the cells to each other should persist at least as long as the cells remain connected by the primary cell connective (spindle remnant or cell bridge), or until other forces move and re-orient them. The physico-chemical nature of the forces that orient the centrioles is as yet unknown (Schrader, 1947, 1953). It is clear that no simple explanation, in terms of electrostatic charges, nor of electromagnetic polarization, can account for rod centrioles orienting at right angles to each other.

A corollary of this hypothesis is the idea (already reasonably well established by others, as a working hypothesis) that the centrioles are the loci of origin of the orienting forces that spin out the astral rays and, at least, the continuous spindle fibers, and give rise to the distal centriole (blepharoplast) of the spermatid which spins out the axial filament of the sperm. There is likewise much evidence that the centrioles are the loci of forces orienting the mitochondria, etc. (Bowen, 1920, pp. 345-348; Johnson, 1931, p. 124).

Obviously, a theory dealing with centriolar behavior can have no direct application to cleavage patterns or cell orientations in forms where centrioles are lacking. (See also footnote 3.)

The apparent exceptions to the corollary view, in the forms where the spindle fibers originate from the kinetochores of the chromosomes (especially in anastral and acentriolar mitoses, and in forms showing diffuse chromosome fibers), may possibly be resolved by the suggestion (made years ago by Darlington, 1936; Schrader, 1936; Pollister, 1939; Pollister and Pollister, 1943; etc.) that the kinetochores contain centriolar material, or are, in effect, centrioles. This was especially beautifully demonstrated by Pollister's work on the supernumerary centrioles of certain gastropods. This matter could be resolved still more simply, if one assumes that the centrioles form the continuous spindle fibers, and the kinetochores, as slightly different counterparts of the centrioles, form the chromosomal fibers.

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[3] It should be kept in mind that the role played by the centers or centrosomes in the mechanism of mitosis may vary in different organisms. Thus, Dietz (1959, *Zeitschr. Naturforsch.*, **14b**: 749–753) has shown that under experimental conditions in the insect, *Pales crocata*, a bipolar spindle may be formed even when one or both centrosomes are located in a distant part of the cell, and anaphase movements of the chromosomes may be quite normal. But how such an abnormal configuration of spindle elements affects cytokinesis is not reported by Dietz. Presumably, in *Pales*, also, irregularities in the division of the cell as a whole will result from such a displacement of the centers. Once the centrioles have done their work in spinning or orienting the fibers necessary for a given division, they may have no further function in that particular mitotic division. Hence, they could be removed and have the division continue. The kinetochores of the chromosomes might assume the entire function of fiber-formation under such circumstances, as they normally do in acentriolar mitosis. A more critical test of the effects of removal of centrioles would be to study the succeeding division (if this takes place) or to study the effect of centriole removal in a form where the centrioles clearly spin out the continuous fibers.

Further possible implications

This relation can be further checked by examination of the spindle axes in cleavage products of various other forms in which the centrioles are visibly rods of sufficient length so that their orientation can be ascertained.

If all the relations of centriolar patterns (it is obvious that there must be more than two) to cleavage patterns could be established, we could then use the more easily determined cleavage pattern as a means of ascertaining the centriolar pattern of the previous cell generation. This would enable us to predict what the centriolar pattern and behavior may be in forms in which the centrioles are too small (or too short, as cylinders) to ascertain this pattern directly. It may be possible, by merely glancing at a section of testis under the low power of the microscope, and finding that the axes of many secondary spermatocytes are at right angles to the axes of their nearest mates, to predict that the centrioles will be at right angles in the primary spermatocytes (as in *Oecanthus*). Where the spindle or cell axes lie parallel in adjacent secondary daughter cells, the *Gelastocoris* type of centriolar pattern must have obtained in the primary spermatocytes.

It is possible, of course, that there may be, in some forms, no constant relation between the daughter cells, but even in this case, the variability of arrangement may be attributable to the centriolar behavior. For example, in *Anasa tristis,* Paulmier (1899) found (p. 243) that ". . . the axes of the daughter spindles [in the two daughter secondary spermatocytes] were rarely parallel—the angles at which they lie being dependent upon the angles of the planes in which the two pairs of centrosomes [centrioles, in present terminology] move apart, these angles varying within 90°."

One further possible function of centrioles in determining movements of or within cells might be revealed through the investigation of the telokinetic movements in spermatids. In the spermatids (of the *Gelastocoris* type), when the centrioles move to one side of their respective nuclei (the same side) in both spermatids, as the interzonal connectives elongate, is there a correlated rotation of the two spermatid nuclei away from each other, one rotating clockwise, the other counterclockwise? Payne's paper does not show this stage.

The centriolar movements are presumably the first indications of the telokinetic movements, which have been described in more detail by Montgomery (1911), Bowen (1922) and Johnson (1931). Johnson (1931) states (p. 126), "Nuclei of sister spermatids may rotate into any position with respect to one another, about 55% turning at right angles to the direction of their sisters; i.e., if one is seen at the side of a spindle remnant, the sister nucleus is seen above or below the remnant. I do not believe that the direction of telokinetic movement in one cell has much effect upon the other product of the same division, but rather that the direction is controlled by the spatial relations of the cyst." But could it not be controlled by the positions of the centrioles in relation to the nuclei of the two daughter cells? Johnson's Figure 42 (Figure 7,H of the present paper) shows that the centriolar complex takes up exactly this position, so that the centriole-nuclear arrangement in one daughter spermatid is the opposite of that in the other. This is exactly opposite to the situation we should expect in *Gelastocoris*. We may tentatively suggest, therefore, that the centrioles supply the motive force that causes the interzonal fibers to continue to expand, to bring about the telokinetic rotation.

Early embryologists made many attempts to correlate the position of the first plane of cleavage or the median plane of the embryo with either the sperm entrance point or the copulation path of the migrating sperm nucleus. In various species these efforts met with some degree of success. The entrance point or sperm path may bear some constant relation, within these species, to the orientation of the sperm centriole, when it is finally in position to produce the centrioles of the sperm diaster, which become the centrioles of the first cleavage spindle. Hence, in this situation, also, the final orientation of the sperm centriole and the axis of separation of the daughter centrioles could be the significant feature in determining the first cleavage plane.

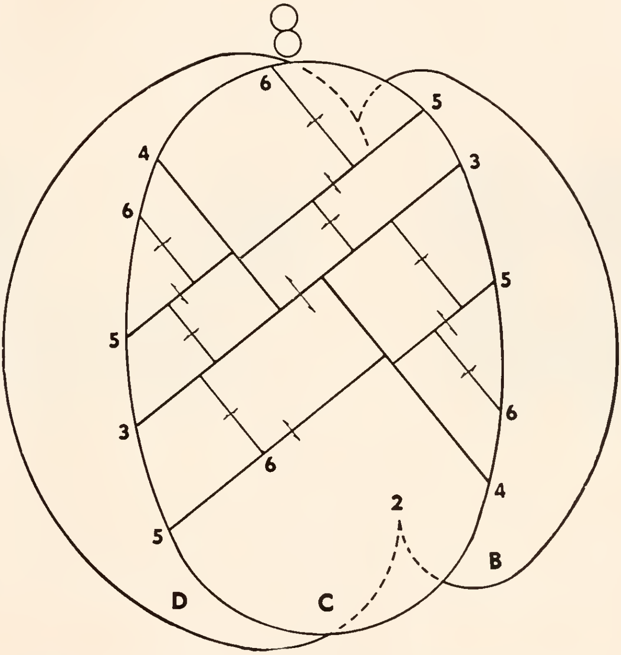


FIGURE 11. Diagram showing the positions of the successive cleavage planes from the third to the sixth (indicated by numbers), projected on the C quadrant of an unequally spirally cleaving egg (such as *Nereis*). Cleavages are alternately dexiotropic (odd-numbered) and leiotropic (even-numbered). Note that all dexiotropic cleavage planes are *at right angles* to all leiotropic planes. The A blastomere is omitted, and the second cleavage plane interrupted, for clarity. Displacements of the cells are not shown.

Patterns and transitions during spiral cleavage

The hypothesis that the orientation and behavior of centrioles may account for the pattern of spiral cleavage in forms like *Polychoerus*, where duets of micromeres are formed, can be readily extended to the quartet type of spiral cleavage, typical of *Crepidula, Nereis, Chaetopterus*, etc. In quartet type spiral cleavage, the spindle of any given cell lies approximately at right angles to those in adjacent cells in both adjoining quadrants at the 4- and 8-cell stages. The successive divisions of each cell, after the 4-cell stage, are alternately dexiotropic and leiotropic, during the entire spiral period of cleavage. However, the first two cleavages in forms such as *Nereis* have the first two cleavage planes somewhat differently directed than the third through the sixth cleavage planes (see Figure 11). In the somewhat simpler situation of spiral cleavage by duets, as exemplified by the *Polychoerus* egg, it is only the first cleavage plane that is slightly anomalous, the others (until the transition to bilateral cleavage begins) being alternately leiotropic and dexiotropic.

There are thus two "critical" periods in spiral cleavage. The first is the period before micromere formation (examples: the first cleavage of *Polychoerus,* and the first two cleavages of *Nereis, Crepidula,* etc.). The second occurs when there is an inevitable transition from the strict alternation of dexiotropic and leiotropic cleavages to bilateral cleavages which herald the production of the bilaterally symmetrical acoel, polyclad, annelidan or molluscan larva. We know that in *Crepidula* (Conklin, 1897) the first cleavage is "prospectively" dexiotropic and the second is "prospectively" leiotropic, as evidenced by the post-cleavage rotations of the protoplasmic areas and by the movements which lead to the formation of the cross-furrow. Perhaps someone will be able to pursue this subject, in a suitable material, and follow the behavior of the centrioles while the egg is undergoing the transition from spiral to bilateral cleavage. To my knowledge, there has been no other hypothesis suggested to attempt to account for the sequence of dexiotropic and leiotropic cleavages.

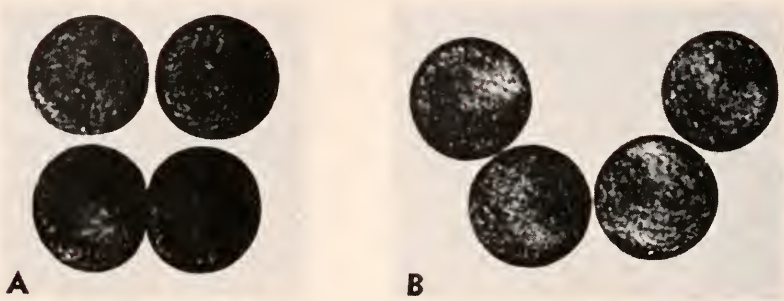


FIGURE 12. Four-cell stages of two living eggs of *Arbacia punctulata.* Magnification 340 ×. These eggs were fertilized in sea water containing Ishida (1936) hatching enzyme. They were transferred to Rulon calcium-low sea water 87 minutes later, and photographed 104 minutes after insemination. Note that the primary cell connective (spindle remnant) of the first cleavage connects the two lower blastomeres, and the two spindle remnants of the second cleavage connect the other daughter cells. All spindle axes are thus clearly indicated. (A) The initial position of the blastomeres. (B) Position after a very gentle tap on the coverglass.

The primary cell connective

As cytokinesis is brought to completion and the two daughter cells are separated by the advancing cleavage furrow, there remains a spindle remnant of continuous and interzonal fibers, sometimes containing Zwischenkörper (Fig. 15). This spindle remnant, together with a thin layer of cortical cytoplasm and/or cell membrane, becomes the stalk or bridge connecting the daughter cells after division is complete. In other words, the spindle remnant constitutes the major portion of the primary cell connective. This connective persists for a considerable period of time, if not permanently, in many materials. This is obvious to anyone who has ever isolated cleavage blastomeres free-hand with fine glass needles; the spindle remnant offers resistance to the needle and must be cut through. The axis of the spindle, of course, determined the point of attachment of the primary cell connective between the daughter cells.

If the eggs of *Arbacia punctulata* are treated to remove the membranes and examined at the 4-cell stage, as earlier demonstrated by Moore, (1930a, 1930b, 1945) for other echinoderm eggs, they show (Fig. 12) the primary cell connectives of the first cleavage spindle between the two middle cells, and the spindle remnants of the two second cleavage spindles at right angles to the first, between the other pairs of cells, exactly as pointed out by Moore (1930a, 1930b) for *Strongylocentrotus* and *Dendraster*. In the echinoderm, which is a radially cleaving form, these connectives are in the same plane, which is not the case in a spirally cleaving egg. The cell connectives are therefore a reflection of the pattern of orientation of the earlier spindles.

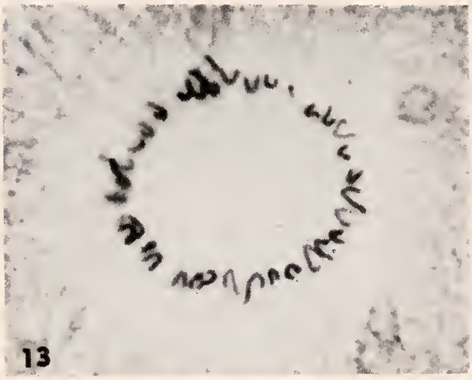
Kojima (1960) has also described the blastomere arrangements in the sea urchin egg, and has experimentally altered this relation by means of dinitrophenol. However, he does not interpret his results in terms of the centrioles, indicating only that the cause of the rearrangement is unknown. For really complex arrangements of multicellular bridges formed from spindle remnants among numerous cells during the oögenesis of *Vespa* (hornet), see the paper by Majiarski (1913).

Sections through the eggs of *Polychoerus* just after the completion of the first division clearly show (Fig. 15) the nature of the primary cell connective. At the point where the furrow membrane meets the spindle remnant, Zwischenkörper may be distinctly seen. These have been described in many other cases, by early workers on cell division.

I believe that the primary cell connectives are far more important than is generally realized. In fact, the persistence of these connectives through a number of cell divisions may be a significant difference between Metazoa and colonial forms, on the one hand, and Protozoa on the other. In certain metazoan blastomeres the cell connectives are apparently totally absent. Fuliński (1916) described the complete separation of the blastomeres of the egg of *Dendrocoelum*, where the individual blastomeres lie dispersed in the external yolk. Here some other factor must have assumed the role taken by the primary cell connective in most forms. That the connectives are absent or can be relatively easily ruptured in some forms is indicated also by the experiments on the mechanical isolation of sponge cells (Wilson, 1907) and their subsequent reaggregation. Presumably, also, in many types of tissue cultures, the cells are free to wander.

Primary vs. secondary forces orienting spindles, and other problems

It is obvious that there are secondary forces which may re-orient a formed or forming spindle, and change the original relations which may have been established by centriolar orientation. The second maturation division of the ovum provides a striking demonstration of this fact. The second maturation spindle (of the secondary oöcyte), formed at the inner end of, and at right angles to, the axis of the first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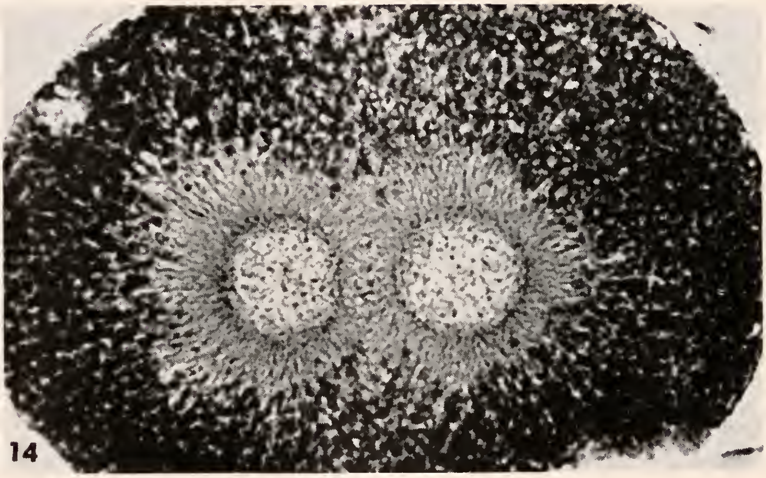
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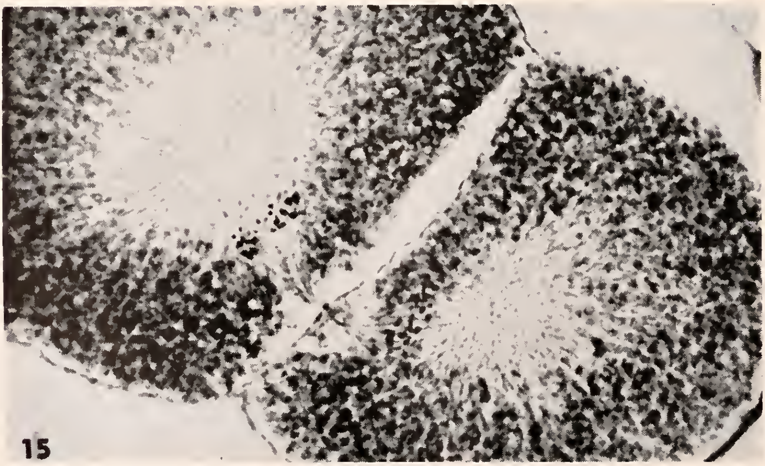
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FIGURES 13 through 15 are of *Polychoerus carmelensis.*

meiotic spindle (the position brought about by the separation of the inner daughter centrioles), must be rotated through 90° to become oriented perpendicular to the egg surface and give off the second polar body under the first. Similar forces may likewise be involved in orienting the first meiotic spindle perpendicular to the egg surface, at the animal pole. The nature of such polar forces is unknown, as is the nature of the forces that orient daughter centrioles with respect to each other. However, the maturation divisions of the ovum are terminal divisions—leading to no future role for the egg centrioles, in those cases in which the entering sperm centriole gives rise to the centrioles of the cleavage diaster. There may be other secondary re-orienting factors as well.

An equally important problem is whether there are differences in centriolar behavior patterns in cases where the daughter cells return to their resting interphase condition, as compared with rapidly dividing cells in which there is no nuclear reconstruction between successive cleavages. Conklin's extensive studies (1902) on centrosome and sphere during the cleavages of *Crepidula* may supply pertinent evidence here.

Division of cleavage centrioles

Because of the relative paucity of *Polychoerus* material available following the resting first cleavage metaphase stage, the stages of centriolar division have not yet been worked out. However, sufficient material was studied to suggest that the whole central apparatus of the *Polychoerus* egg may follow a pattern similar to that described by Vejdovský and Mrázek (1903) for the egg of the oligochaete, *Rhynchelmis*. In *Polychoerus* (Fig. 14), shortly after the eggs are laid, ". . . drastic changes occur at the central region of each aster, while the chromosomes are still at metaphase. The centriole disappears and the centrosomal region becomes a large sphere, which has acquired a definite boundary, not traversed by the rays, which remain outside it. Internally, the sphere is reticular, with numerous tiny granules, no one of which can be identified as a centriole. These spheres (centrospheres, astrospheres, or centrosomes), 40 μ or more in diameter, with their surrounding rays, are beautifully described as 'polar suns.'" (Costello, 1960a). In *Rhynchelmis*, there is a stage in the centrosomal cycle exactly corresponding to this "polar sun" stage. It is of transitory duration, and new daughter centrioles arise from within the reticulate mass where the old centriole had disappeared. The "disappearance" of a centriole may mean only that it has become unstable or unstainable, and has disappeared as a microscopically visible particle. Its reappearance may mean only that it has again become stainable, or of a size large enough to be microscopically detectable. This may complicate the study of the division cycle of the cleavage centrioles in *Polychoerus* by light microscopy, but it in no way negates the hypothesis outlined above.

FIGURE 13. Polar view of metaphase plate of resting first cleavage metaphase, showing all 34 chromosomes. Two were cut and one fragment of each was in the adjacent section. Fixation: Worcester's; stain, Heidenhain's iron haematoxylin. 990 ×.

FIGURE 14. Reticulate centrospheres and surrounding asters of 8-micron sagittal section of metaphase of egg just laid. Fixation: Flemming's; stain: Flemming's tricolor. 480 ×.

FIGURE 15. Zwischenkörper, spindle remnant, chromosome vesicles, etc., at late telophase of first cleavage, in 8-micron section. Fixation: Flemming's; stain: Flemming's tricolor. 480 ×.

SUMMARY

1. The centrioles of the egg of *Polychoerus carmelensis,* at first meiotic metaphase, second meiotic metaphase, and resting first cleavage metaphase, are slightly curved rods which are usually oriented at right angles to each other and to the main axis of the spindle.

2. Centriole orientation and behavior in the spermatocyte divisions of Gryllidae and Hemiptera, as described by Johnson (1931) and Payne (1927), in relation to the arrangements of daughter cells, are compared with centriole orientation and predicted behavior in the egg of *Polychoerus.*

3. These considerations (on centriole orientation and behavior) constitute the basis for a new hypothesis, as follows:

a. The orientation of the centrioles at any given division determines the position in which the daughter centrioles will separate from each other.

b. The path of separation of daughter centrioles determines the position of the main axis of the spindle for the next division.

c. The axis of the spindle determines the relative positions of the daughter cells with respect to each other.

d. This arrangement of the daughter cells is maintained, for a time at least, by the primary cell connective, of which the spindle remnant is the significant portion.

e. These relations obtain in the absence of secondary intervening factors.

4. The inherent right-angle orientation of the centrioles at the two poles of the first cleavage spindle of *Polychoerus* is thus interpreted in causal relation to the alternating dexiotropic and leiotropic divisions in spiral cleavage.

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A CYTOCHEMICAL INVESTIGATION OF OÖGENESIS AND DEVELOPMENT TO THE SWIMMING LARVAL STAGE IN THE CHITON, CHITON TUBERCULATUM L.[1]

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The chitons have received very little attention from modern developmental biologists. Older accounts dealing with the morphology of chiton development have been published by Heath (1899), Hammersten and Runnström (1925), and Grave (1932). Yet for the chemical embryologist this group offers a number of advantages. Chitons may be readily induced to shed gametes in the laboratory, and artificially inseminated eggs develop well under laboratory conditions. Large numbers of eggs are produced by each individual, and although these eggs are surrounded by a rather ornate chorion, only a thin layer of jelly is present. Development in the species used in this study is quite rapid, requiring only about 12 hours at 26° C. to produce a swimming trochophore. In addition, like all molluscs, chitons are mosaic and exhibit the classical spiral pattern of cleavage.

The objective of this investigation has been to examine the pattern of distribution of nucleic acids, proteins and mucopolysaccharides during the development of the gonadal oöcyte, and during the subsequent development of the egg into the swimming larva. Since the mosaicism of the chiton egg must be dependent upon its physico-chemical organization, by examining oögensis cytochemically one might hope to identify those aspects of the organization of the egg which are causally related to the mosaic nature of its development.

Further, a consideration of the distribution of the macromolecular substances during development could yield information about the patterns of synthetic activity in molluscan mosaic development as compared to mosaic development in other phyla and also to regulative development.

Materials and Methods

The chitons used in these studies were collected in the vicinity of the Bermuda Biological Station, St. George's West, Bermuda, and identified as *Chiton tuberculatum* L. Specimens were removed from intertidal rocks at low tide and maintained in moist but not filled sea water tanks until the second evening after their collection. Individual animals were then placed in fingerbowls and covered with

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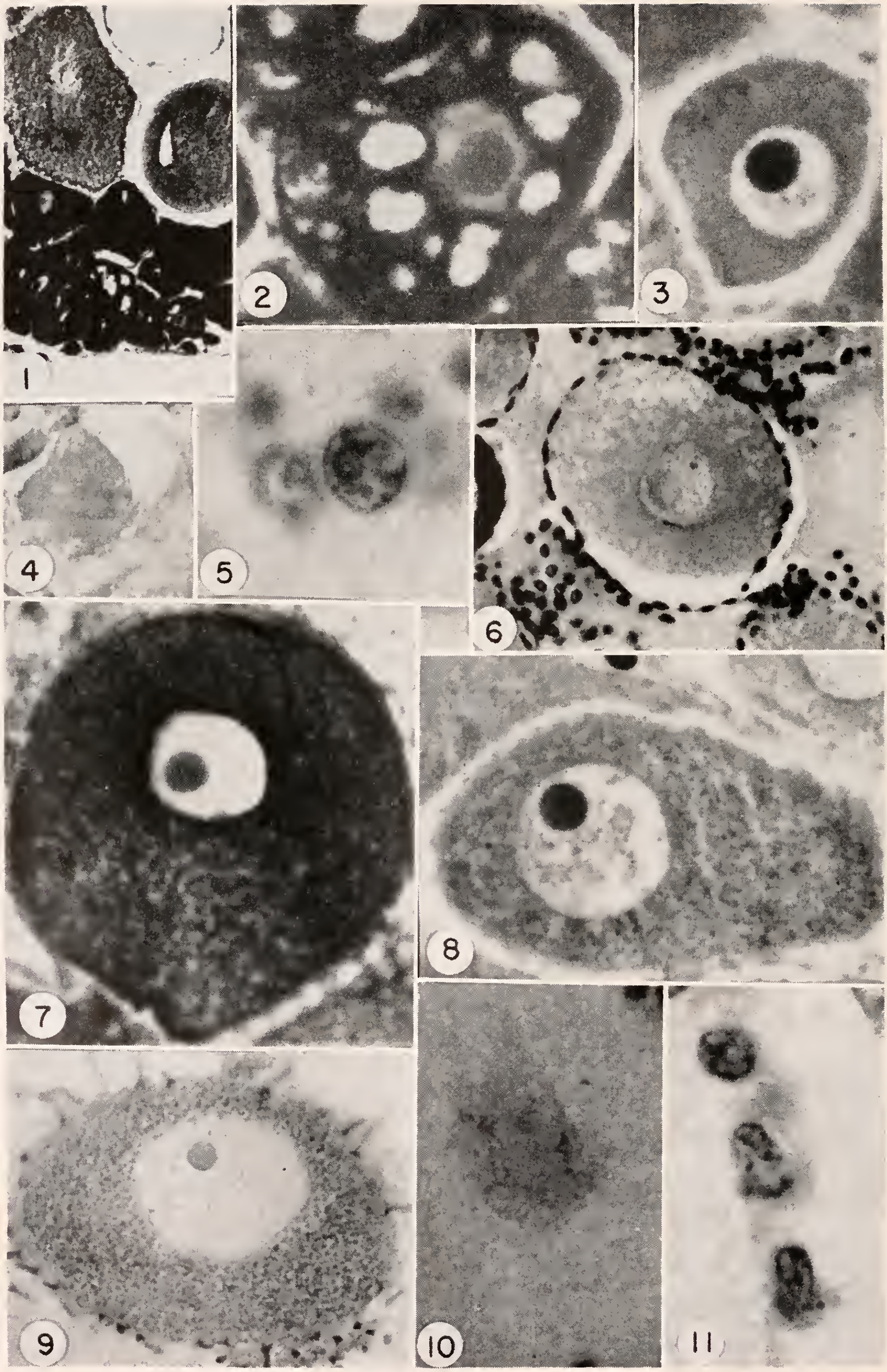
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FIGS. 1–11.

sea water. Ovulation or the release of sperm ensued two to three hours later. Eggs were then inseminated with sperm suspension and allowed to develop. Development was observed with a binocular dissecting microscope, and embryos were removed for fixation at the desired stages.

For studies of oögenesis, the ovaries were removed from fresh specimens and fixed in appropriate fixatives. Acetic acid-ethanol (1:3) fixation was used for material to be stained for nucleic acids, Lillie's (1954) calcium acetate formalin for material to be stained for mucopolysaccharides and proteins, and Bouin's for material to be stained in mercuric bromphenol blue for proteins.

The pH 4.0 azure B bromide method of Flax and Himes (1952) was used for demonstrating RNA in sections which had been pre-treated with DNAase; Einarson's (1949) gallocyanin-chromalum method was used for staining DNA in sections which were pretreated with RNAase. Crawley *et al.*'s (1956) alcian blue-periodic acid Schiff (AB-PAS) method, which distinguishes between acid mucopolysaccharides and other polysaccharides or substances containing 1,2-glycol groups, was used for visualizing mucopolysaccharides. Some sections of embryos were stained with either alcian blue or with the PAS reaction alone. Some control sections were incubated in 0.1% α-amylase prior to staining with the PAS reaction to remove glycogen. Mazia, Brewer and Alfert's (1953) mercuric bromphenol blue (MBB) method for reactive groups of proteins was employed on Bouin-fixed sections. The picro-naphthol blue black-brilliant purpurin-azofuchsin (NBB-BP-Az) connective tissue method of Lillie (1954) was used without the conventional nuclear staining on calcium acetate formalin-fixed material in an attempt to distinguish between various protein components of the cells. Each of the three acid dyes used in this mixture stains only protein structures, probably by binding to the sites of dissociable basic groups. Some calcium acetate formalin-fixed sections were stained for protein tryptophan by Adam's (1957) dimethylaminobenzaldehyde method.

The eggs and embryos were partially dehydrated in 70% and 95% ethanol and then dehydrated in tertiary butanol. They were infiltrated with first a slush of

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FIGURE 1. Chiton ovary, low power photomicrograph. Azure B bromide after DNAase. 300 ×.

FIGURE 2. Early stage one oöcyte with large but not heavily basophilic nucleolus. Azure B bromide after DNAase. 1920 ×.

FIGURE 3. Stage one oöcyte. Mercuric bromphenol blue. 1920 ×.

FIGURE 4. Early stage one oöcyte showing almost complete absence of cytoplasmic staining. Protein tryptophan method. 750 ×.

FIGURE 5. Smallest recognizable oöcyte stained for DNA; note nucleolus-associated heterochromatin and coarse nuclear chromatin. Gallocyanin-chromalum after RNAase. 4800 ×.

FIGURE 6. Larger stage one oöcyte surrounded by accessory cells. Gallocyanin-chromalum after RNAase. 1200 ×.

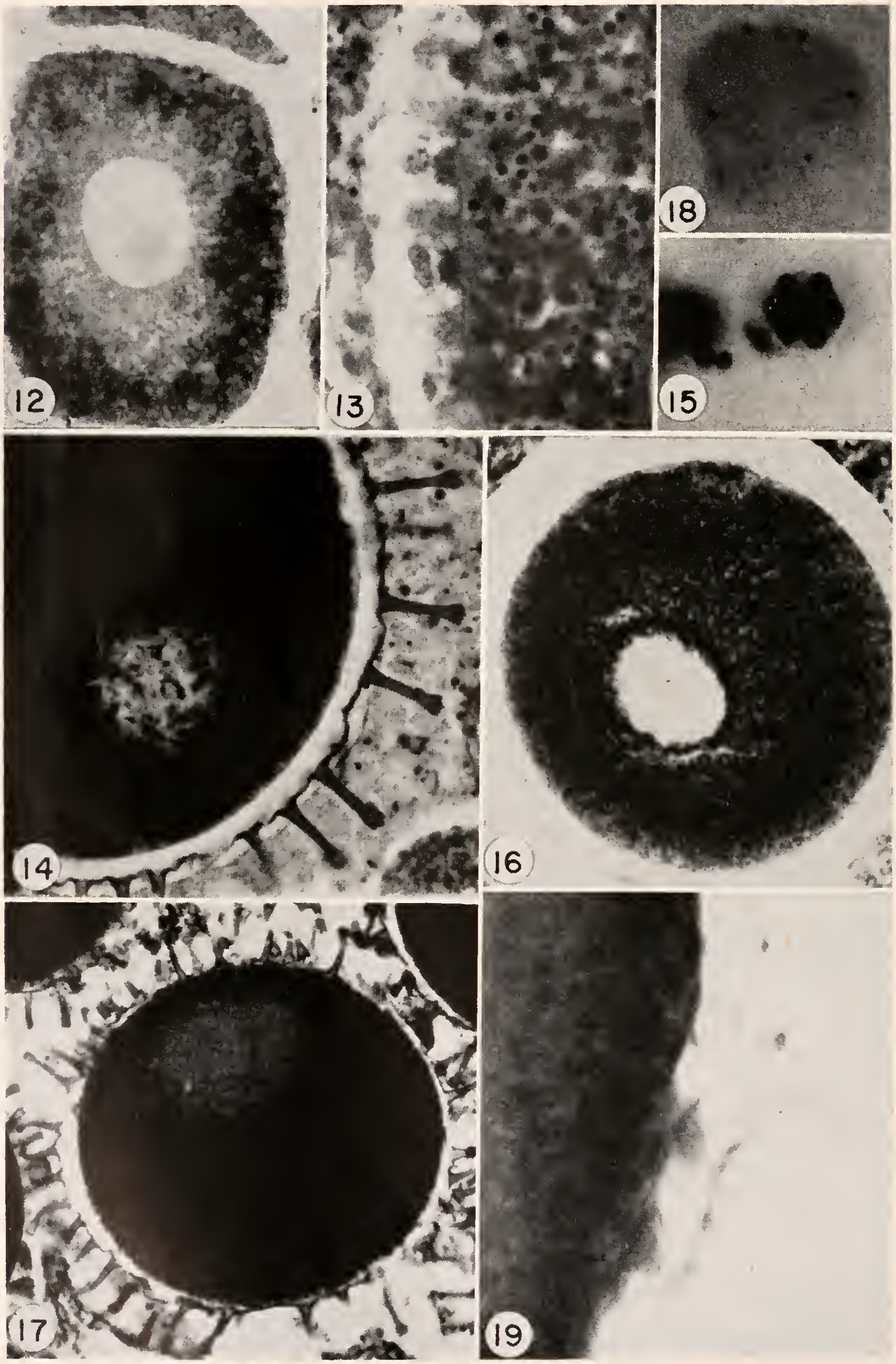
FIGURE 7. Stage two oöcyte with somewhat diminished cytoplasmic basophilia and relatively smaller nucleolus than stage one oöcytes. Azure B bromide after DNAase. 1200 ×.

FIGURE 8. Stage two oöcyte showing distribution of proteins. Mercuric bromphenol blue. 1200 ×.

FIGURE 9. Terminal oöcyte showing nucleolus and protein yolk granules stained by protein tryptophan method. 768 ×.

FIGURE 10. Stage two oöcyte nucleus in pachytene. Gallocyanin-chromalum after RNAase. 1200 ×.

FIGURE 11. Nuclei of oöcyte accessory cells. Gallocyanin-chromalum after RNAase. 4800 ×.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FIGS. 12–19.

equal parts of tertiary butanol and Tissuemat, and finally in pure Tissuemat. This treatment is preferable to conventional infiltration with xylol or toluol for yolky material. Eggs, embryos and ovaries were embedded in 56–58° C. m.p. Tissuemat and serially sectioned at 5 μ except for the specimens to be stained for DNA, which were sectioned at 15 μ.

OBSERVATIONS

Oögensis. A germinal epithelium surrounds the chiton ovary. The smallest oöcytes were found on the periphery of the ovary; the oöcytes increase in size toward the inner portion of the ovary (Fig. 1). Three different stages could be recognized in the development of the chiton oöcytes. In the first stage the oöcyte underwent its greatest relative increase in size, and this was also the stage in which the principal elaboration of cytoplasmic RNA occurred; in the second stage yolk synthesis was initiated and a chorion was formed. Except for the nuclei which were in diplotene and diakinesis of meiosis, third stage oöcytes were indistinguishable from freshly ovulated eggs.

The cytoplasm of stage one oöcytes was intensely basophilic. The nuclei contained a single basophilic nucleolus which occupied a large proportion of the nuclear volume (Fig. 2). In sections stained with mercuric bromphenol blue for basic groups of proteins, the relative intensity and distribution of staining were similar to that of basophilia due to RNA (Fig. 3). Differential staining of various protein structures was obtained with the (NBB-BP-Az) method: nucleoli were stained red by azofuchsin, nuclear proteins brown by brilliant purpurin, and cytoplasmic proteins red by azofuchsin. Toward the end of the first stage, however, there was a change in the staining behavior of the oöcyte cytoplasmic proteins. Instead of binding the red dye, azofuchsin, they bound the brown dye, brilliant purpurin. Again the distribution of staining was similar to that of RNA basophilia. The protein tryptophan method produced only weak staining in the cytoplasm of stage one oöcytes (Fig. 4). The cytoplasm of these oöcytes also contained very little glycogen; the cytoplasm was faintly PAS-positive, and this could be abolished by prior digestion in α-amylase. The oöcyte nucleoli were slightly PAS-positive, and nucleolar staining persisted with this method through all three stages until the nucleolus disappeared. No alcian blue staining was observed in stage one oöcytes which could not be prevented by prior incubation in RNAase.

FIGURE 12. Chiton oöcyte showing distribution of alcian blue-stainable material (dark) and PAS-positive material (light) at stage of chorion formation. Alcian blue-PAS. 1920 ×.

FIGURE 13. Evagination of chorionic processes from chorion, pushing accessory cells away. Mercuric bromphenol blue. 3000 ×.

FIGURE 14. Chorionic processes fully developed with "stalk" and terminal "bud." Picronaphthol blue black-brilliant purpurin-azofuchsin. 1200 ×.

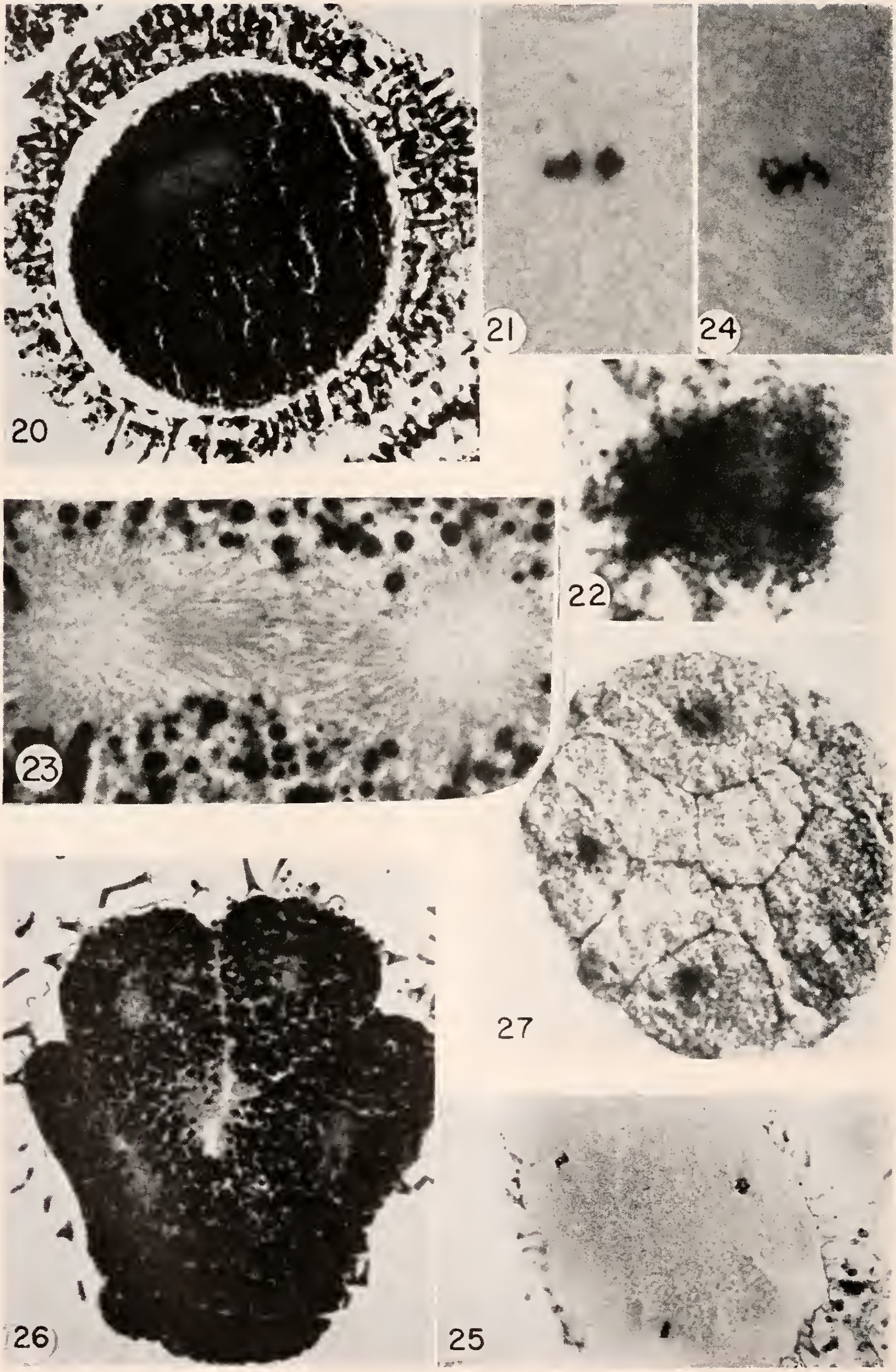
FIGURE 15. Top view of "bud," showing six radially symmetrical points and a center point. Picronaphthol blue black-brilliant purpurin-azofuchsin. 4800 ×.

FIGURE 16. Stage three oöcyte, showing reduced cytoplasmic basophilia except around nuclear membrane and absence of nucleolus. Azure B bromide after DNAase. 1600 ×.

FIGURE 17. Stage three oöcyte: cytoplasm is filled with uniform protein yolk granules. Mercuric bromphenol blue. 600 ×.

FIGURE 18. Stage three oöcyte nucleus stained for DNA, showing postpachytene chromosomes adherent to nuclear membrane. Gallocyanin-chromalum after RNAase. 1920 ×.

FIGURE 19. Cortical layer of stage three oöcyte is stained by alcian blue, the endoplasm beneath is filled with PAS-positive protein yolk granules. Alcian blue-PAS. 4800 ×.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FIGS. 20–27.

The chromosomes were somewhat condensed in very small stage one oöcytes. The nucleolus was surrounded by nucleolus-associated heterochromatin which, like the chromosomes, was stained by the gallocyanin-chromalum method for DNA (Fig. 5). In the later phases of stage one, the oöcytes became surrounded by a layer of somewhat flattened oöcyte accessory cells (Fig. 6).

In the second stage of oöcyte growth the concentration of cytoplasmic basophilia was reduced and the nucleolus occupied a relatively smaller proportion of the nucleus (Fig. 7). In addition to the RNA-associated proteins which were present in stage one, small granules of protein yolk appeared which were deeply stained by mercuric bromphenol blue (Fig. 8). With the (NBB-BP-Az) method, the yolk granules were stained by azofuchsin while the RNA-associated proteins were stained by brilliant purpurin; both the nucleolus and nucleoplasm continued to stain as in stage one. The yolk granules were strongly stained by the protein tryptophan method (Fig. 9), and were also strongly PAS-positive. Again, within the oöcyte there was no alcian blue staining which was not removable by prior digestion in RNAase. The stage two oöcyte chromosomes were in pachytene, and were adherent to the nuclear membrane (Fig. 10).

The second stage oöcytes were chiefly concerned with yolk synthesis, but outside the oöcyte, the oöcyte accessory cells were involved in the construction of a chorion. While these cells were somewhat flattened in stage one, they proliferated and eventually formed a single layer of rounded cells around the stage two oöcyte (Fig. 11). A chorionic membrane was then laid down around the oöcyte beneath the oöcyte accessory cells. The chorion itself was PAS-positive (Fig. 12) and was lightly stained by both mercuric bromphenol blue and by azofuchsin in the (NBB-BP-Az) method. Then from beneath the oöcyte accessory cells, hollow hydranth-like processes evaginated from the chorion (Fig. 13). These processes grew outward from beneath the oöcyte accessory cells. When fully developed, the processes consisted of a distal hydranth-like "bud," a stalk, and a basal region which opened into the space between the chorion and the oöcyte (Fig. 14). The "bud" consisted of six radially symmetrical points and one center point (Fig. 15). When the development of the processes was completed, the accessory cells were no longer in evidence.

The cytoplasm of stage three oöcytes was considerably less basophilic than the cytoplasm of stage two oöcytes. The basophilia was distributed between the large protein yolk granules except in the region of the nuclear membrane where it seemed to be accumulated in a concentric pattern (Fig. 16). The nucleoli had disappeared

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FIGURE 20. Ovulated egg. Picronaphthol blue black-brilliant purpurin-azofuchsin. 768 ×.

FIGURE 21. Polar body division stained for DNA. Gallocyanin-chromalum. 4800 ×.

FIGURE 22. Polar body division, showing staining of the meiotic apparatus and fine cytoplasmic granules surrounding the apparatus. Mercuric bromphenol blue. 4800 ×.

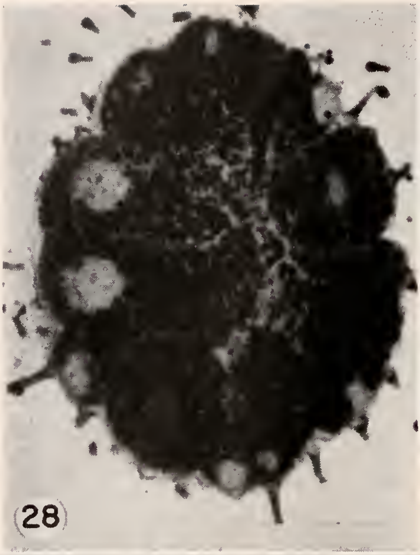
FIGURE 23. Mitotic apparatus of an early cleavage figure stained for protein. Mercuric bromphenol blue. 3840 ×.

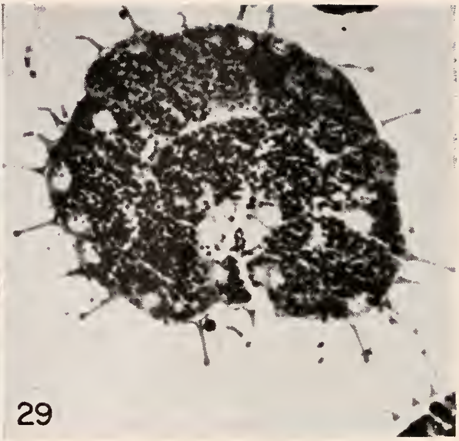
FIGURE 24. Cleavage figure stained for DNA; note spindle is longer and narrower than polar body spindle. Gallocyanin-chromalum after RNAase. 4800 ×.

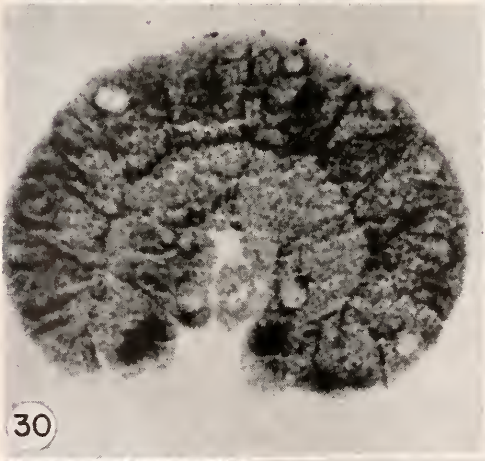
FIGURE 25. Spiral cleavage pattern. Gallocyanin-chromalum after RNAase. 800 ×.

FIGURE 26. Early cleavage embryo stained for protein; note equal density of protein yolk granules in all blastomeres. Mercuric bromphenol blue. 768 ×.

FIGURE 27. Intermediate cleavage stained for RNA; note equal distribution of cytoplasmic basophilia; dark nuclear zones are mitotic apparati. Azure B bromide after DNAase. 768 ×.


28


29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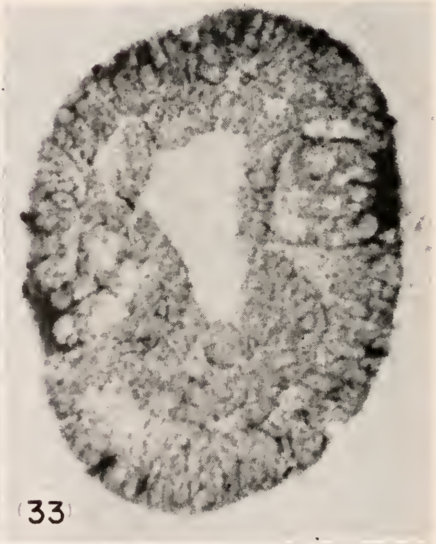
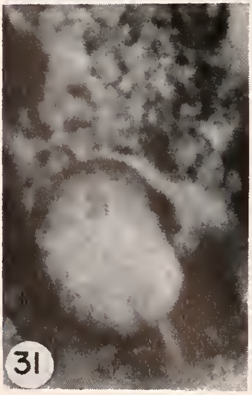
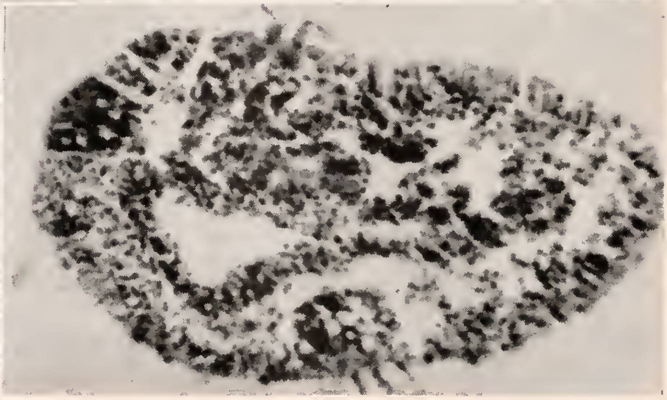

33


31



Figs. 28–33.

in most stage three oöcyte nuclei. The cytoplasm was filled with protein yolk granules of uniform size which gave staining reactions identical to those observed in the smaller growing granules of stage two (Fig. 17). In the nucleus, the nucleolus had disappeared, and the post-pachytene chromosomes were observed adherent to the nuclear membrane (Fig. 18). Beneath the chorion, a granular cortical zone appeared which was stained by alcian blue (Fig. 19), presumably acid mucopolysaccharide material.

The chorionic processes had completed their outward growth by the beginning of stage three, and the oöcyte was coated with mucoprotein material. This material surrounded the oöcyte from the chorion to just beyond the distal "buds" of the chorionic processes (Fig. 20). This substance was lightly stained by both mercuric bromphenol blue and azofuchsin in the (NBB-BP-Az) method, faintly PAS-positive, and contained some granules which were deeply stained by alcian blue.

Early development. After ovulation, the first meiotic division occurred (Fig. 21). Shortly after fertilization, a second division followed, but it was not possible to determine the sequential relationship between the second polar body division, and the entrance of the sperm. The cytoplasmic distribution of both protein yolk granules and RNA-basophilia was identical with that observed in stage three oöcytes except in the region of the meiotic apparatus. Here yolk granules were pushed aside and the spindle was surrounded by smaller granular protein material (Fig. 22). Basophilia was associated with the spindle.

The mitotic apparati of cleaving embryos were stained by the mercuric bromphenol blue method for proteins (Fig. 23). Cleavage occurred by normal mitosis (Fig. 24), and exhibited the spiral pattern (Fig. 25). Both protein yolk and basophilia were evenly distributed within the cells (Figs. 26 and 27). Since the macromeres were larger they received more material, but not through any obvious differential segregation of material. No nucleoli were formed in the cleavage blastomere nuclei. However, there was considerable difference in the size of macromere and micromere nuclei (Fig. 28).

By the time of gastrulation the density of protein yolk granules was greater in the endodermal cells which had been derived from the invaginated macromeres (Fig. 29). No local increases in basophilia were observed (Fig. 30), but very small nucleoli appeared in the ectodermal cells which later became the prototroch or apical tuft. These nucleoli were only faintly basophilic in the gastrula (Fig. 31). The slight difference in degree of basophilia between the two primary layers is probably due to differences in cell size.

FIGURE 28. Intermediate cleavage stained for proteins; note equality of distribution of protein yolk granules and disparity in size of nuclei of the various blastomeres. Mercuric bromphenol blue. 768 ×.

FIGURE 29. Gastrula stained for protein; somewhat heavier concentrations of protein yolk granules in the endoderm. Mercuric bromphenol blue. 600 ×.

FIGURE 30. Gastrula stained for RNA; with the exception of division figures, basophilia not significantly different in the primary layer. Azure B bromide after DNAase. 768 ×.

FIGURE 31. Prototroch cell showing increased cytoplasmic basophilia and a small nucleolus. Azure B bromide after DNAase. 4800 ×.

FIGURE 32. Trochophore larva showing distribution of protein; note yolk in the larval gut. Picronaphthol blue black-brilliant purpurin-azofuchsin. 768 ×.

FIGURE 33. Trochophore larva showing distribution of RNA; the prototroch and apical tuft cells are basophilic. Azure B bromide after DNAase. 768 ×.

In the swimming larva, protein yolk was most dense in the endodermal cells of the trochophore gut (Fig. 32). The cytoplasm of the cells of the prototroch and apical tuft was intensely basophilic, and their nucleoli were somewhat more heavily stained than in the gastrula (Fig. 33). In the other cells of the trochophore, there was no local increase in basophilia, and no nucleoli were present.

Discussion

The patterns of appearance and distribution of RNA in chiton oöcytes were similar to those described in other molluscan species (see Raven, 1958). The greatest relative volume increment occurred in the first stage of oöcyte growth. This was also apparently the stage in which the principal synthesis of cytoplasmic RNA occurred. In the successive stages of oöcyte growth cytoplasmic basophilia decreased, suggesting that either RNA synthesis had ceased entirely or that the rate of its synthesis was not commensurate with the oöcytes' growth in volume. The oöcyte nucleoli had also reached their maximum size and intensity of basophilia by the end of the first stage. Ranzoli (1953) reported a similar situation in the oöcytes of the gastropod *Patella*. RNA synthesis and nucleolar growth apparently reached a peak just prior to the initiation of protein yolk synthesis. In all of the published accounts of RNA distribution in relation to oöcyte growth in molluscs, a progressive decrease in cytoplasmic concentration has been noted as the oöcytes increased in size. RNA synthesis appears to be chiefly restricted to the first stage of oöcyte growth prior to protein yolk synthesis in *Chiton tuberculatum*, and if some cytoplasmic RNA synthesis continues after the nucleoli start to become depleted, as suggested by Swift *et al.* (1956), it is not sufficient to maintain cytoplasmic RNA concentrations at the level observed in the first stage of oöcyte growth during the subsequent stages. Further, the results of this investigation suggest that very little RNA synthesis occurs during the development of the chiton to the trochophore stage. The little synthesis that did occur was limited to a single kind of cell: namely, the cilia-bearing cells of the prototroch and apical tuft.

The single incidence of such a pattern of RNA distribution during oögenesis and early embryology would hardly represent sufficient grounds for proposing a general mechanism in mosaic development. Recently, however, Cowden and Markert (unpublished data) have investigated the distribution of RNA during the development of ascidian, *Ascidia nigra*, and here also no evidence of new synthesis of RNA was found at any stage from the second stage of gonadal oöcyte growth to the initiation of metamorphosis in the swimming larva. This is not true of all molluscan or ascidian embryos. Minganti (1950) reported that basophilia increased during the development of the gastropod *Lymnaea*, and according to Raven (1958), nucleoli initially appear in the nuclei of 24-cell stage embryos of *Lymnaea*. More recently Mancuso (1959) has described the appearance of nucleoli and increasing cytoplasmic basophilia in cells taking part in major morphogenetic events such as neural tube formation or myogenesis in the ascidian, *Ciona*. In mosaic development, however, primary differentiation does not occur at or just prior to gastrulation, but is fixed at the latest by the four-cell stage. While it is probable that the new synthesis of RNA may be initiated at any time during the later course of development, starting earlier in some species than in others, the production of new RNA does not appear to play any role in primary differentiation of molluscan or ascidian embryos. To do so, synthesis would have to be initiated before the second cleavage, and none of the usual manifestations of RNA synthesis have been detected in early cleavage stages in any of the molluscan or ascidian embryos for which adequate cytochemical studies are available. If RNA has any function in primary differentiation, it must depend on RNA produced during oöcyte growth. Such a function could be possible if there were a heterogeneity in the distribution of qualitatively different kinds of RNA in the mature ovum. In this way the different kinds of RNA could be segregated into the blastomeres at cleavage. Many of the classical experiments on the effects of centrifugation on the development of mosaic eggs suggest that some form of cytoplasmic "structuring" is present in these eggs.

Protein yolk synthesis occurred primarily in the second stage of chiton oöcyte growth. These protein yolk granules were considerably richer in protein tryptophan than the other cytoplasmic proteins. Thus the synthetic activities of the oöcyte fell into two distinct phases: the elaboration of RNA-proteins and the production of specialized cytoplasmic proteins. Since the oöcyte is a very specialized cell, this dichotomy may not necessarily represent the patterns of synthetic activity of cells in general, but is probably true of other cell types which produce a particular kind of protein in quantity, *e.g.*, the mammalian reticulocyte. It is also interesting to note that the staining affinity for acid dyes of the cytoplasmic RNA-associated proteins was altered as the oöcytes approached the period of protein yolk synthesis. Since these dyes in acid solutions are generally bound at the sites of dissociated basic groups of proteins, such a shift in affinity would not be expected to depend on differences in kinds of reactive groups but rather on differences in the arrangement of dissociated reactive groups. According to Baker (1958), differential binding of acid dyes in mixtures probably depends on the size of the dye molecule, the spacing of its reactive groups, the degree to which the dye molecules tend to form aggregates, and the structural arrangement of the proteins in question. A shift in affinity, such as that observed in the later stage one chiton oöcytes, could indicate an alteration in the structure of the RNA-protein complex. Both Himes (1958) and Love and Liles (1959) have developed methods for detecting differences in the RNA-protein complex by alterations in the capacity of the RNA-moiety to bind basic dyes under certain conditions. Such alterations can also apparently be detected by staining methods designed to attach to the protein moiety. By the end of the first stage, the generation of cytoplasmic ribonucleoprotein (RNP) had essentially been completed, and an alteration in the RNP complex in preparation for protein synthesis might occur. This is particularly interesting in the light of recent electron microscope studies which have indicated that Golgi zones might be specializations of the endoplasmic reticulum. Bolognari (1960) has convincingly demonstrated that protein yolk synthesis in the oöcyte of two molluscan species occurs within the Golgi zones, and that these Golgi zones are composed of concentric lamallae which contain dark granules of about 300 Å diameter.

The functions of the oöcyte accessory cells and chorionic processes deserve some comment because of their unusual nature. The processes themselves seem to be hollow modifications of the chorion which presumably increase its surface area and facilitate exchange. The oöcyte accessory cells were involved in the construction of the process since each process grew outward into an accessory cell and the accessory cells were absent when the processes were completely formed. The oöcyte accessory cells were also probably involved in the construction of the chorion. When the accessory cells achieved their maximum size and number, the chorion appeared between the accessory cells and the cortex of the oöcyte. The role of these cells may not necessarily involve the production of chorionic material since there was an abundance of cortical PAS-positive and protein material in the oöcyte, but they may produce a tanning agent which hardens the chorionic material. In other invertebrates which have tough chorions such a mechanism is involved. Monné (1955) and Smyth (1956) have investigated chorion formation in a number of invertebrates, and some form of quinone tanning was involved in each case.

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SUMMARY

1. Oöcyte growth and development and the subsequent development of the egg to the swimming larva stage were investigated in the chiton, *Chiton tuberculatum*, using topological cytochemical methods for nucleic acids, proteins and mucopolysaccharides.

2. The growing oöcytes exhibited two phases of synthetic activity: the first phase was chiefly concerned with RNA production and the second phase with protein yolk synthesis.

3. A probable alteration in the cytoplasmic RNA-protein complex prior to the initiation of yolk synthesis was detected by an alteration of the affinity of RNA-associated proteins for binding acid dyes in mixtures.

4. Oöcyte accessory cells were probably responsible in some way for chorion formation. After the chorion was formed, ornate hollow processes grew out of the chorion, each beneath an accessory cell. When development of the processes was completed, the accessory cells had disappeared.

5. There was no evidence of new synthesis of RNA in any of the cells of the developing embryo except the cells of the prototroch and apical tuft. This applies to the complete developmental history of the oöcyte from the cessation of RNA synthesis prior to protein yolk formation in the oöcyte to the swimming trochophore larva.

6. Since primary differentiation occurs in mosaic embryos in the absence of synthesis of new RNA, the possibility that primary differentiation is controlled by the RNA produced in the oöcyte was discussed.

7. Although there was no differential segregation of protein yolk into the blastomeres—both RNA-basophilia and protein yolk being evenly distributed in all blastomeres—the posterior blastomeres received a larger proportion of yolk material than the micromeres. This was particularly evident in the yolk-laden cells of the trochophore gut.

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PHYSIOLOGY OF THE VINEGAR EEL, TURBATRIX ACETI (NEMATODA). I. OBSERVATIONS ON RESPIRATORY METABOLISM[1]

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The demonstration of the Krebs tricarboxylic acid cycle in many vertebrate tissues, and failure to confirm reports of its absence in others, have led to the conclusion that the cycle is probably ubiquitous among this group of animals (Krebs, 1954). There is also evidence for the operation of the cycle, or for one or more reactions of the cycle, in several groups of invertebrates which have been studied (*ibid;* Read, 1960). However, some of the trypanosomes lack the tricarboxylic acid cycle (von Brand, 1957) and this pathway is reported to be absent in *Trichomonas vaginalis* (Wirtschafter *et al.,* 1956; Read, 1957) although the closely related species, *T. gallinae,* possesses enzymes which oxidize the intermediates of the cycle (Read, 1957).

The present communication reports some studies which indicate that the vinegar eel (*Turbatrix aceti*), a free-living nematode, may lack the tricarboxylic acid cycle in its usual form.

MATERIALS AND METHODS

Culture and preparation of vinegar eels

The vinegar eels used in these experiments were grown from cultures obtained from Turtox Biological Supply Company. The organisms were grown by inoculating commercial clear apple juice diluted 1:1 with water with a dense suspension of eels in vinegar (about 20 ml. per quart of diluted apple juice). Other fruit juices, *e.g.,* pear, peach, apricot, may also be used although the sediment interferes with observation and cleaning of the worms. The inoculated juice was poured into gallon bottles to a depth of about one inch. The bottles were covered loosely and kept at room temperature. After 7–10 days the fermenting cultures were dense with vinegar eels. At this point the bottles were half-filled with vinegar. These cultures could be kept for long periods of time with no attention. If the fermenting juice was kept too long without adding vinegar, putrefaction with loss of the culture often occurred. With a little experience it was possible to judge the right time for the addition of the vinegar. For some experiments reported the organisms were grown by inoculation into a medium containing peptone (Difco) 0.5%, yeast extract (Difco) 0.3%, NaCl 0.5%, and acetic acid 2–4%. The pH was about 3 to 3.5, depending on the amount of acetic acid added. This medium offers no advantages over vinegar, and the establishment of a dense culture requires several months.

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[1] Part of this work was carried out in the Zoology Department, University of California, Los Angeles, California, during the tenure (senior author) of a predoctoral Fellowship from the National Institutes of Health, U. S. Public Health Service, and constituted a portion of a thesis submitted to the Graduate Division in partial fulfillment of the requirements for the Ph.D. degree.

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Several methods of concentrating the organisms and washing to free them of the bulk of contaminating organisms were used. The following method, which was found to be the most satisfactory, depends on the negative geotropism of the vinegar eels. The cultures in vinegar or other medium are subjected to a preliminary concentration in a separatory funnel. Most of the organisms will, within a few minutes, aggregate at the surface of the liquid in a dense layer ¼ inch or so deep. The lower liquid is drained off rapidly. The concentrated suspension remaining is poured into burettes (50- or 100-ml.). After a few minutes the lower portion of the burette will contain few worms and the organisms will have begun to aggregate at the surface. The stopcock is opened slightly and the liquid allowed to drain out dropwise at a rate which will leave the surface-aggregated organisms behind, adhering to the wall of the burette. If the outflow rate is properly adjusted, most of the vinegar eels will remain on the walls of the burette when the liquid has drained out. The worms are rinsed from the burette with a small amount of a suitable solution (*e.g.,* 3% acetic acid or 0.05 *M* phthalate buffer, pH 3.0). When a sufficient number of worms has been collected they may be washed any desired number of times by repetition of this procedure or by brief centrifugation (500 × G). The latter procedure is not recommended except for the final washings and packing of the organisms, since repeated centrifugation damages the worms to the extent of interfering with their normal negative geotropic behavior.

For some of the experiments reported the organisms were prepared by washing them two or three times as above, holding them for 24 hours in a non-nutrient medium (0.05 *M* phthalate, pH 3.0), re-washing as above, and finally washing three times by centrifugation in the medium in which the organisms were to be finally suspended or homogenized.

In earlier experiments (the determination of substrate oxidation by intact organisms), where it was necessary to free the worms as completely as possible from contaminating micro-organisms, the vinegar eels were prepared by centrifuging two or three times with mechanical removal of the bulk of contaminating micro-organisms which form a layer above the denser eels. Following this initial washing, the suspension was placed in a Buchner-type funnel with a sintered glass disc of medium porosity through which 100–200 ml. of 0.05 *M* phthalate, pH 3.0, were allowed to run over a period of several hours. Final concentration of the organisms was achieved by centrifugation in all cases. Rapid attainment of the relatively low centrifugal force (about 500 × G) necessary for sedimentation of the organisms and rapid deceleration are essential for satisfactory sedimentation. The total time needed, from starting to stopping, is less than two minutes. Longer centrifugation injures the worms and slow stopping allows time for redispersion. The most satisfactory instrument for the purpose is a small table-top, swinging cup centrifuge. This type rapidly attains maximum speed and can be stopped quickly.

Determination of oxygen consumption

In a typical manometric experiment with intact *T. aceti,* 2.7-ml. portions of a dense suspension of organisms (giving a reading of about 100 units with a #54 green filter in the Klett-Summerson colorimeter against a water blank) in the appropriate buffer were pipetted into Warburg flasks of 12–18-ml. capacity. Folded filter paper and 0.2 ml. of 30% KOH were placed in the center wells. Substrate or inhibitor dissolved in buffer (0.3 ml.), to give the final concentration indicated, was pipetted into the side bulb. Following an equilibration period, readings of endogenous oxygen consumption were taken at 15-minute intervals for a two-hour period. Substrate (or inhibitor) was then added from the side bulb and the oxygen consumption measured during another two-hour period. Control flasks with buffer alone in the side bulb were included to correct for changes in endogenous oxygen consumption. In some of the experiments reported the side bulb contents were tipped in at the beginning of the measurement period. Experiments with intact organisms were carried out at 24.5° C. The gas phase was air. The system was buffered with 0.05 *M* phthalate except where otherwise noted. At the conclusion of an experiment the total nitrogen content of each flask was determined by the method of Johnson (Umbreit, Burris and Stauffer, 1949). Oxygen consumption is reported as μl. of oxygen consumed per mg. of tissue nitrogen per hour (Q_{O_2} (N)) except in the experiments with fluoracetate where the total oxygen consumed in 200 minutes is reported.

Enzyme assays

Homogenates were prepared in all-glass homogenizers immersed in ice water. Homogenates were prepared on the basis of the volume of packed vinegar eels following a two-minute centrifugation at about 500 × G. Thus a 5% homogenate was made by dispersing 0.2 ml. of packed organisms in 4 ml. of homogenizing medium. Homogenization performed intermittently for 5 to 8 minutes yielded a milky suspension almost free of intact worms. Homogenates for manometric measurement of succinic or malic dehydrogenase were prepared, in different experiments, in water, 0.154 *M* KCl or in 0.5 *M* sucrose. For the spectrophotometric assays using phenazine methosulfate as an electron carrier, the homogenates were prepared in 0.5 *M* sucrose. Homogenates for measurement of oxidation of keto acids were made in 0.154 *M* KCl containing 8 ml. of 0.04 *M* $KHCO_3$ per liter.

Succinic dehydrogenase was assayed in homogenates by three methods. (1) The manometric method of Schneider and Potter (Umbreit, Burris and Stauffer, 1949). (2) The Thunberg method: the system contained 0.03 *M* sodium phosphate, 0.045 *M* sodium succinate, 0.05 mg./ml. methylene blue, pH 7.4, total volume 5.0 ml. After evacuation and equilibration at 37° C., one ml. of a 10% *T. aceti* homogenate in water was added from the side bulb of the Thunberg tube. At intervals during a two-hour period at 37° the optical density of the tubes was measured at 600 mμ. (3) A colorimetric method employing phenazine methosulfate (PMS) to mediate electron transfer between the enzyme and dichloroindophenol (Ells, 1959). The assay system contained 0.05 *M* potassium phosphate, 0.01 *M* KCN, 2.3×10^{-5} *M* sodium 2,6-dichloroindophenol, 0.02 mg. PMS, and 0.01 *M* sodium succinate. The pH of the system was 7.4 and the total volume was 6.0 ml.

The change in the optical density of the system was followed spectrophotometrically at 610 mμ. Succinate oxidized was assumed to be equal, on a molar basis, to dichloroindophenol reduced.

Malic dehydrogenase was tested for by the method of Potter (Umbreit, Burris and Stauffer, 1949) and by an adaptation of the colorimetric PMS method. The reaction mixture for the latter assay was the same as the system for succinic dehydrogenase except that 3.3×10^{-4} *M* pyridine nucleotide (DPN or TPN) was added and 0.008 *M* sodium DL-malate was substituted for succinate. Sodium fumarate (0.0067 *M*) was added in addition to or in place of malate in some assays.

TABLE I

Effect of 0.05 M acetate on respiration of intact T. aceti. The pH of the system was 3.0. Time refers to the time lapse between removal of the organisms from the original culture and beginning of the measurement of respiration. Figures starred are averages of duplicates, the others of triplicates.

Time (hours)	$Q_{O_2}(N)$		% Change
	Endogenous	With acetate	
3	142	189	+33
24	72*	335*	+365
39	62	136	+119

Attempts were made to demonstrate isocitric dehydrogenase in *T. aceti* homogenates in a system containing 0.05 *M* Tris, 0.04 *M* sodium DL-isocitrate, 0.001 *M* $MnSO_4$, 2.3×10^{-5} *M* sodium 2,6-dichloroindophenol, 0.02 mg. phenazine methosulfate and either 6.7×10^{-4} *M* each of DPN and AMP or 6.7×10^{-4} *M* TPN. The pH was 7.4. The optical density at 610 mμ was measured at 30-second intervals for at least 10 minutes in all experiments.

The oxidation of α-keto acids (pyruvic, oxalacetic and α-ketoglutaric) was measured manometrically by the method of Potter, Pardee and Lyle (Umbreit, Burris and Stauffer, 1949).

Citrate synthesis was measured following incubation of a suspension of intact vinegar eels at room temperature in 0.02 *M* sodium acetate, pH 4.5. At the end of the incubation period 1.0-ml. aliquots of the suspension were rapidly homogenized and deproteinized by the addition of 1 ml. of 5% sodium tungstate and 1 ml. of 0.33 *M* H_2SO_4. Citrate was determined in the filtrates by the method of Taussky and Shorr (1947).

RESULTS

Endogenous oxygen consumption and oxidation of acetate

Suspensions of vinegar eels in phthalate or phosphate buffer show $Q_{O_2}(N)$ values of about 50 to 200, depending on the time lapse between removal of the organisms from a nutrient culture medium and measurement of oxygen consumption. The upper value is approached by organisms used within two or three hours of removal from culture while the lower values are obtained with organisms maintained in a non-nutrient solution for 36 hours or more.

Acetate markedly stimulates the respiration of vinegar eels, as shown by the data in Table I. Following a three-hour period without exogenous substrate, while the endogenous respiration is still high, acetate produces only a slight stimulation of oxygen consumption. As the endogenous respiration falls, the stimulatory effect

TABLE II

Effect of Krebs cycle intermediates on the respiration of intact T. aceti. Respiration was measured at the pH indicated as described in the Methods section. "Before" and "after" refer to respiration before and after tipping in the contents of the side bulb (buffer alone or buffer with substrate), time as in Table I. In some experiments side bulb contents were tipped into flasks at the beginning of the measurement period. Figures starred are averages of duplicates, others of single determinations.

Substrate in side bulb	Final conc. (*M*)	Time (hrs.)	Q_{O_2}(N)								
			pH 3.0			pH 4.5			pH 6.0		
			Before	After	% Chg.	Before	After	% Chg.	Before	After	% Chg.
None	—	4	—	164*	—	—	—	—	—	—	—
Citrate	0.05	4	—	167*	—	—	—	—	—	—	—
None	—	8	—	232*	—	—	—	—	—	—	—
Citrate	0.05	8	—	247*	—	—	—	—	—	—	—
None	—	23	100	89	−11	80	74	−8	82	99	+21
Citrate	0.05	23	125*	117*	−7	96*	89*	−7	93*	93*	0
None	—	48	66	81	+23	70	81	+16	63	79	+25
Citrate	0.01	48	74*	81*	+9	65*	81*	+25	61*	82*	+34
None	—	4	—	164*	—	—	—	—	—	—	—
Succinate	0.05	4	—	191*	—	—	—	—	—	—	—
None	—	8	—	232*	—	—	—	—	—	—	—
Succinate	0.05	8	—	210*	—	—	—	—	—	—	—
Succinate	0.05	17	96*	80*	−10	126*	123*	−2	120*	107*	−11
None	—	48	51	67	+31	93	93	0	45	68	+51
Succinate	0.01	48	70*	89*	+27	85*	85*	0	57*	79*	+39
None	—	4	—	164*	—	—	—	—	—	—	—
DL-malate	0.05	4	—	100*	—	—	—	—	—	—	—
None	—	8	—	232*	—	—	—	—	—	—	—
DL-malate	0.005	8	—	216*	—	—	—	—	—	—	—
None	—	23	100	89	−11	80	74	−8	82	99	+21
DL-malate	0.05	23	104*	107*	+3	104*	97*	−7	98*	96*	−2
None	—	48	66	81	+23	70	81	+16	63	79	+25
DL-malate	0.01	48	85*	98*	+15	81*	94*	+16	69*	95*	+38
None	—	16	—	—	—	59*	73*	+24	74*	86*	+16
Oxalacetate	0.05	16	50*	108*	+116	71*	121*	+72	97*	143*	+47

Table III

Effect of malonate on respiration of intact T. aceti. Respiration was measured at pH 4.5. Figures starred are averages of duplicates, the others of single determinations.

Acetate (M)	Malonate (M)	Q_{O_2}(N)		% Change
		Before addition	After addition	
0	0.001	65*	89*	+37
0	0.02	71	103	+45
0.01	0	53*	116*	+119
0.01	0.001	65*	130*	+100
0.01	0.02	61*	149*	+144
0.05	0	48	166	+246
0.05	0.001	46*	175*	+280
0.05	0.02	57*	170*	+198

of acetate increases. The explanation for the very much higher acetate stimulation in worms which had been maintained in non-nutrient medium for 24 hours than in worms three hours out of the original culture is not known. This kind of effect was fairly regularly observed although it was not always so striking. The lower effect of acetate on the respiration of organisms which have been substrate-depleted for much longer periods of time (39 hours in Table I) is also characteristic and perhaps reflects irreversible damage to the metabolic machinery.

Effect of Krebs cycle intermediates on respiration of intact organisms

Several intermediates of the Krebs cycle were added to suspensions of intact *T. aceti*, usually after preliminary measurement of endogenous respiration. In some experiments the endogenous respiration and the respiration in the presence of substrate were measured simultaneously in different flasks. Usually two substrate concentrations were used and organisms exhibiting two or more levels of endogenous respiration were tested with each substrate. Each substrate was tested at pH 3.0, 4.5 and 6.0. The results are shown in Table II. Under the conditions employed, citrate, succinate and malate appear to be without significant effect on oxygen consumption compared with the control flasks in which buffer without substrate was added from the side bulb. Oxalacetate, however, does stimulate respiration. The increased effect of this compound at lower pH is probably due to

Table IV

Effect of fluoracetate on the respiration of intact T. aceti. Respiration was measured in 0.05 M phosphate with 0.05 M acetate, pH 7.3. In each experiment the suspension of organisms was adjusted to give a reading of 130 units in the Klett-Summerson colorimeter with a #54 filter.

µl./200 minutes

No fluoracetate	0.01 M fluoracetate
52.9	27.1
73.2	37.7
57.6	29.8

more rapid penetration of the organisms by undissociated oxalacetic acid than by oxalacetate ion.

Effect of malonate on respiration of intact organisms

Table III summarizes data on the effect of malonate on the respiration of intact *T. aceti*. Endogenous oxygen consumption is not inhibited by malonate at concentrations of 0.001 and 0.02 *M*, and there may be a slight stimulation of respiration although the number of determinations is too small to establish this with certainty.

Citrate synthesis and the effect of fluoracetate

Fluoracetate (0.01 *M*) was found to inhibit the oxygen consumption of intact vinegar eels, as shown in Table IV. This suggested that a pathway for the synthesis of citrate and aconitase, or some other fluoracetate-sensitive enzyme catalyzing the further metabolism of citrate might be operative in these organisms. Intact *T. aceti* which had been maintained several hours in a non-nutrient medium were found to synthesize citrate when acetate was added to the medium (Table V). Furthermore, the accumulation of citrate under these conditions was increased in the presence of 0.01 *M* fluoracetate.

TABLE V

Synthesis of citrate by T. aceti in the presence and in the absence of fluoracetate. Incubations performed in 0.02 M sodium acetate, pH 4.5.

Experiment	Incubation period	Citrate synthesized (μg./mg. (N))	
		No fluoracetate	0.01 *M* fluoracetate
1	30 mins.	11.0	11.0
2	2 hrs.	12.4	41.2
3	8 hrs.	30.2	176.0
4	8 hrs.	93.4	181.3

Substrate oxidation by T. aceti homogenates

Several attempts were made, without success, to demonstrate succinic dehydrogenase manometrically in homogenates of vinegar eels fortified with cofactors necessary for respiration by homogenates of mammalian tissues. The reaction mixture included mammalian cytochrome *c* to serve as an electron carrier. Rat liver homogenate controls consumed oxygen rapidly in this system. Experiments using the Thunberg technique showed that *T. aceti* homogenates did not reduce methylene blue anaerobically in the presence of succinate.

Neither cytochrome *c* nor methylene blue can accept electrons directly from mammalian succinic dehydrogenase (Singer and Kearney, 1954) but only from other electron carriers which are able to react with the enzyme. Phenazine methosulfate is the most active artificial electron acceptor which will react directly with succinic dehydrogenase. When this dye was used, with dichloroindophenol as the

TABLE VI

Oxidation of succinate by T. aceti homogenate using phenazine methosulfate as an electron carrier

System	Succinate oxidized (mμmoles/min./ml. 5% homogenate) Experiment 1	Succinate oxidized (mμmoles/min./ml. 5% homogenate) Experiment 2
Complete	7.6	5.5
Minus succinate	0.6	0.0

terminal electron acceptor, a low succinic dehydrogenase activity was demonstrated in vinegar eel homogenates (Table VI).

T. aceti homogenates containing DPN, cytochrome *c* and other cofactors necessary for the demonstration of malic dehydrogenase in mammalian tissues did not consume oxygen in the presence of malate. Rat liver homogenate controls showed a high activity. When PMS was used to couple electron transfer between reduced pyridine nucleotides and dichloroindophenol, malate was rapidly oxidized by *T. aceti* homogenates in the presence of DPN (Table VII). TPN would not replace DPN, and fumarate at a concentration nearly equal to that of the DL-malate in the system did not inhibit. When fumarate was used as substrate in this system instead of malate, the rate of reduction of dichloroindophenol was less than 10% of that with malate, indicating that the fumarase activity is very low.

There was no oxygen consumption by vinegar eel homogenates in the presence of pyruvate, oxalacetate or α-ketoglutarate and the cofactors necessary for the oxidation of these keto acids by mammalian tissue homogenates. Isocitric dehydrogenase activity could not be demonstrated in *T. aceti* homogenates using either TPN and Mn^{++} or DPN and AMP as cofactors. PMS, which will accept electrons from either DPNH or TPNH, was used as an electron carrier in these experiments.

DISCUSSION

The experiments with homogenates, together with the finding that several of the Krebs cycle intermediates do not stimulate the respiration of intact *T. aceti*, suggests that the cycle, in its usual form, does not operate in this species. Stimula-

TABLE VII

Oxidation of malate and fumarate by T. aceti homogenate using phenazine methosulfate as an electron carrier

Substrate	Pyridine nucleotide	Substrate oxidized (mμmoles/min./ml. of 5% homogenate) Experiment 1	Substrate oxidized (mμmoles/min./ml. of 5% homogenate) Experiment 2
DL-malate	DPN	152	140
Fumarate + DL-malate	DPN	—	144
Fumarate	DPN	8.2	13.0
None	DPN	0	—
DL-malate	None	2.4	—
DL-malate	TPN	4.7	3.5
DL-malate (no PMS)	DPN	9.4	—

tion of the respiration of intact worms by oxalacetate, demonstration of citrate synthesis, and the inhibition of respiration by fluoracetate indicate that citrate synthetase and aconitase may be present. Isocitric dehydrogenase is apparently absent and the activities of succinic dehydrogenase and fumarase are so low under the conditions of the assay (compared with malic dehydrogenase) as to raise doubt that they function in the main pathway of oxidative metabolism. Indeed, it is not certain that these activities are not due to unavoidable contamination of the homogenates with micro-organisms, although this seems doubtful. Failure of homogenates to oxidize the keto acids under the conditions employed is not conclusive evidence for the inertness of these compounds in *T. aceti* since PMS was not used as electron carrier in these systems.

In the experiments with intact vinegar eels the possibility that a high endogenous respiration might mask the effect of oxidizable Krebs cycle intermediates has been obviated by using organisms at several time intervals after removal from a nutrient medium. The effect on intact organisms of utilizable substrates might be thought to depend on the pH at which measurements are made, as has been shown with *Euglena* (Danforth, 1953). However, in the present study changes in pH over the range 3.0 to 6.0 have no effect on respiration in the presence of the compounds tested, with the exception of oxalacetate. Failure of malate to stimulate the respiration of intact vinegar eels under these conditions is difficult to understand in view of the active malic dehydrogenase these organisms possess. It may be that intact *T. aceti* are impermeable to some organic acids at all pH values. This possibility admittedly casts doubt on conclusions concerning the absence of metabolic reactions based on the failure of compounds to stimulate respiration of intact organisms of this species.

The finding that malonate has no inhibitory effect on acetate oxidation by intact vinegar eels and slightly increases the endogenous respiration may further confirm the thesis that the main pathway of acetate oxidation does not go through succinic dehydrogenase. However, this is not conclusive evidence because of the above-mentioned permeability problems. Read (1956) showed a slight stimulation of the respiration of intact *Hymenolepis diminuta* by malonate, although in cell-free preparations malonate inhibited the oxidation of succinate.

It is clear from the experiments with homogenates, and especially strikingly illustrated in the case of the malic dehydrogenase experiments, that *T. aceti* cannot use mammalian cytochrome *c* in electron transport. Lack of cytochrome *c* reductase or cytochrome oxidase may explain this. Studies of the electron transport system in *T. aceti*, to be published elsewhere, indicate that the latter enzyme, at least, is absent.

Ascaris, a nematode whose oxidative metabolism has been studied in some detail, shows similarities to *T. aceti*. Although a number of intermediates of the citric acid cycle fail to stimulate the respiration of intact *Ascaris* (Rathbone, 1955), fumarase and malic decarboxylase are present (Saz and Hubbard, 1957) and a succinic dehydrogenase has been partially purified (Bueding *et al.*, 1955). Cytochrome *c* and cytochrome oxidase are absent (Bueding and Charms, 1952; Rathbone, 1955). The malic decarboxylase, which oxidatively decarboxylates L-malate to pyruvate, requires either DPN or TPN, the latter being less active (Saz and Hubbard, 1957).

The role of the malic dehydrogenase in *T. aceti* remains in doubt pending further study. It is possible that this enzyme will prove to be a decarboxylase.

SUMMARY

1. Suspensions of intact *Turbatrix aceti* in phthalate or phosphate buffer consume oxygen at a rate varying from about 50 to 200 μl./mg. (tissue nitrogen)/hr. The rate depends on the time lapse between removal of the organisms from the original culture and the beginning of the measurement, *i.e.*, the time without exogenous substrate.

2. Oxygen consumption of intact organisms is markedly stimulated by acetate and also by oxalacetate.

3. Citrate, succinate and malate do not stimulate the oxygen consumption of intact *T. aceti* at pHs 3.0, 4.5 or 6.0 and when tested with organisms showing several levels of endogenous respiration.

4. Malonate at a concentration of 0.001 *M* or 0.02 *M* does not inhibit the endogenous oxygen consumption of intact *T. aceti* nor does it prevent the stimulation of respiration by acetate.

5. Citrate is synthesized by intact organisms in the presence of acetate. Fluoracetate increases this synthesis and inhibits the respiration of intact vinegar eels.

6. Pyruvate, α-ketoglutarate, succinate, malate and oxalacetate are not oxidized by homogenates of *T. aceti* fortified with cofactors necessary for oxidation of these compounds by mammalian liver preparations.

7. When phenazine methosulfate is used to transfer electrons from pyridinenucleotides to dichloroindophenol, an active DPN-malic dehydrogenase can be demonstrated in homogenates. TPN has little or no activity. When fumarate is used in place of malate in this system, activity is low, indicating a low fumarase activity.

8. Using phenazine methosulfate to accept electrons from the enzyme, with dichloroindophenol as terminal electron acceptor, a low succinic dehydrogenase activity can be shown in *T. aceti* homogenates.

9. Isocitric dehydrogenase was not detected in a system containing phenazine methosulfate and dichloroindophenol as electron acceptors and either TPN and Mn^{++} ions or DPN and AMP as cofactors.

10. These findings suggest that, in addition to the active malic dehydrogenase, *T. aceti* possesses citrate synthetase and aconitase but apparently has limited ability to carry out other reactions of the tricarboxylic acid cycle. The electron transport system, unlike that of many invertebrates, cannot utilize mammalian cytochrome *c*.

11. If these interpretations are borne out, *T. aceti* will be the first animal with a predominantly aerobic metabolism in which the tricarboxylic acid cycle in its usual form has been shown to be absent.

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PHYSIOLOGY OF THE MELANOPHORES IN THE CRAB SESARMA RETICULATUM[1]

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In 1948 Brown noted that the black pigment in all crabs, which had been observed, concentrated maximally after eyestalk removal if the animals were not exposed to excessively high intensities of illumination. Later, however, Enami (1951) reported that the pigment in melanophores of specimens of *Sesarma intermedia, S. haematocheir,* and *S. dehaani* dispersed maximally after eyestalk ablation.

Regulation of color change has been studied in a detailed manner in very few species of crabs. In the fiddler crab, *Uca pugilator*, the species whose chromatophores have been studied most intensively, the melanophores are controlled by pigment-dispersing and pigment-concentrating substances (Carlson, 1936; Sandeen, 1950; Brown and Fingerman, 1951; Fingerman, 1956a). A black-pigment-dispersing principle has been found also in the crabs *Hemigrapsus oregonensis* by Bowman (1949), *Callinectes sapidus,* the blue crab, by Fingerman (1956b), *Eriocheir japonicus* by Matsumoto (1954), *Uca rapax* by Burgers (1958), and *Macropipus vernalis* by Burgers (1959). No evidence, however, has been adduced for a melanin-concentrating principle in these crabs. Enami (1951) reported that extracts of central nervous organs from the three species of *Sesarma* evoked concentration of melanin when injected into eyestalkless individuals.

The responses of melanophores to changes in light intensity and temperature have been observed in *Uca pugilator* (Brown and Sandeen, 1948) and *Callinectes sapidus* (Fingerman, 1956b). An albedo response operated to disperse the black pigment when specimens of both species were placed on a black background and to concentrate this pigment in crabs on a white background. Enami (1951) stated that background responses were not exhibited by the three species of *Sesarma* which he observed. With respect to temperature, the melanin in *Uca* tended to concentrate as the temperature increased above or decreased below 15° C. In contrast, the black pigment in *Callinectes* became progressively more concentrated as the temperature increased from 10° to 28° C.

The present investigation was undertaken primarily to expand the small amount of information available concerning chromatophores of brachyurans other than *Uca*, and secondarily to compare the behavior of the melanophores in specimens of *Sesarma reticulatum* from the Woods Hole area with the findings reported by Enami (1951) for three species of *Sesarma* in Japan. Problems such as the chromatics of eyestalkless specimens, background responses, and endocrine regulation of the melanophores were considered.

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MATERIALS AND METHODS

Adult specimens of the grapsoid crab *Sesarma reticulatum* were collected at West Falmouth, Massachusetts, for use in this investigation. In the laboratory crabs were maintained under constant illumination in aquaria containing sea water approximately 2 cm. deep. The intensity of illumination incident on these crabs was 40 ft. c.

Melanophores on the walking legs were observed with the aid of a stereoscopic dissecting microscope and transmitted light and were staged according to the system of Hogben and Slome (1931). Stage 1 represented maximal concentration of the melanin, stage 5 maximal dispersion, and stages 2, 3 and 4 the intermediate conditions.

Tissue extracts were prepared in sea water as described in detail by Sandeen (1950). The dose of each extract injected into assay animals was 0.05 ml. The concentration of each extract was one-third of a complement per 0.05 ml. Eyestalkless specimens, some of which served as assay animals, had had both eyestalks removed at least 12 hours prior to use. The wounds were cauterized to minimize loss of blood.

Student's t test was used for determination of the level of significance between the means. The 5% level was considered the maximum for a significant difference. Standard deviations and standard errors for the differences between means were also calculated.

EXPERIMENTS AND RESULTS

Rhythm of pigment migration

Twenty intact specimens were placed into a darkroom during the afternoon of July 19, 1960. At midnight the average stage of the melanophores of 10 specimens was determined and the crabs were returned to the darkroom. The same procedure was followed at 8:00–8:30 AM, noon–1:00 PM, 5:00 PM, and midnight Eastern Daylight Saving Time for the next seven days.

The means are presented in Figure 1. Inspection of this figure reveals that the melanin was more dispersed by day than at night. The chromatophore indices determined at midnight and at 8:00–8:30 AM for the entire series of observations were compared to determine the statistical significance of the difference between the means. This difference was highly significant.

Relationship between light intensity, background, and chromatophore stage

The objectives of this set of experiments were to determine during the day phase and the night phase of the cycle of pigment migration (1) if specimens of *Sesarma reticulatum* exhibit a background response and (2) the response of the melanophores to changes in total intensity of illumination. For the first experiment 10 intact, recently collected specimens were placed into each of six black and six white pans at 8:00 AM. The animals in one black and one white container then were exposed to one of these intensities of illumination, 2, 17, 40, 70, 250, and 1110 ft. c., for two hours at which time the melanophores of each crab in the 12 containers were staged. This experiment was repeated on another day with one change in the procedure; the crabs were placed under the various intensities at noon instead of at 8:00 AM.

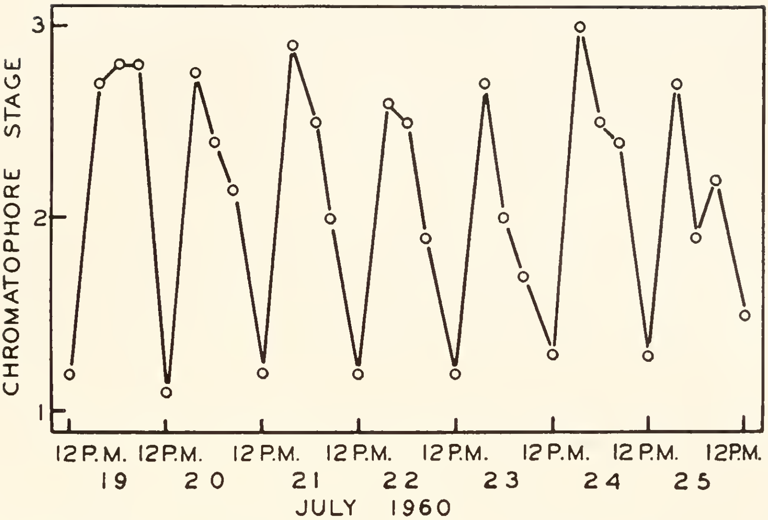

FIGURE 1. Relationship between melanophore stage and time of day in crabs maintained in darkness.

The next two experiments were performed at night between 10:45 PM and 3:00 AM. Ten intact crabs were placed into each of four black and four white pans. The crabs in one black and one white container were then exposed to one of these light intensities, 2, 32, 280, and 1110 ft. c., for two hours. The chromatophore stage of each crab was then determined. This experiment was also performed one more time.

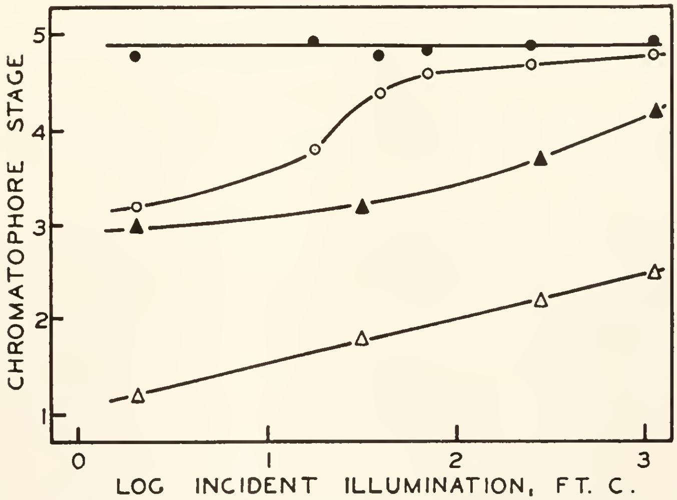

FIGURE 2. Relationships between melanophore stage and the logarithm of the incident light intensity for crabs in the day phase of the rhythm and on a black background (dots), day phase and on a white background (circles), night phase and on a black background (solid triangles), night phase and on a white background (empty triangles).

The means of the data obtained from these experiments were used in the preparation of Figure 2. Inspection of this figure reveals several facts. During the daytime the pigment in the melanophores of crabs in black containers was nearly maximally dispersed at each light intensity used in the experiments. For example, the mean chromatophore stages were 4.75 at 2 ft. c. and 4.95 at 1110 ft. c. Statistical analysis revealed that the difference between these means was not significant.

The melanophores of the crabs in white containers, however, behaved differently. At 2 ft. c. during the daytime the pigment was in an intermediate state (mean stage, 3.15). With increased illumination the degree of pigment dispersion increased with the result that the mean chromatophore stage of the crabs exposed to 1110 ft. c. was 4.80. The difference between these means (3.15 and 4.80) was statistically significant.

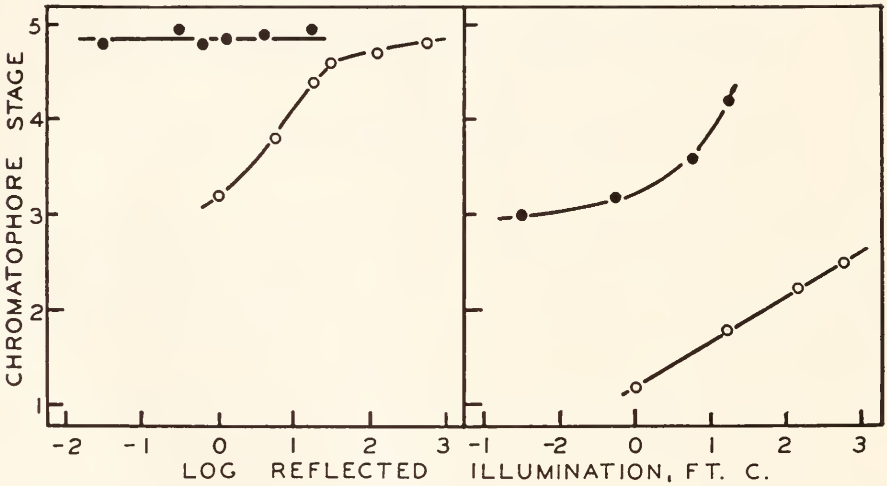

Figure 3. Relationships between melanophore stage and the logarithm of the reflected light intensity for crabs in the day phase (left portion of the figure) and night phase (right portion of the figure) of the rhythm. Circles, on a white background; dots, on a black background.

With regard to the experiments performed at night, crabs in the black and in the white pans darkened in response to increased illumination. The increased dispersion of pigment with increased light intensity in crabs on both backgrounds was statistically significant. The data obtained at 2 and 1110 ft. c. were used in these analyses.

Inspection of Figure 2 reveals that, in addition to the response to incident illumination, the melanin was more dispersed in crabs on a black background than on a white one, with the possible exception of the crabs exposed to 1110 ft. c. by day. The difference between the mean chromatophore stages of the crabs on the black and the white backgrounds during the daytime at an illumination of 2 ft. c. was statistically significant but not at 1110 ft. c. However, the differences between the crabs on the two backgrounds during the nighttime were significant at both 2 and 1110 ft. c.

The findings presented in Figure 2 bear out the cycle of pigment migration shown in Figure 1. At each light intensity used the pigment of crabs on both backgrounds was more dispersed by day than at night. Statistical analysis revealed that the differences between chromatophore stages obtained at 2 and 1110 ft. c. during the nighttime and daytime were highly significant.

To demonstrate clearly that specimens of *Sesarma reticulatum* show a true background (*i.e.*, albedo) response and not merely a response to the amount of light reflected from the background, the data of Figure 2 were replotted in terms of the intensity of light reflected from the black and the white backgrounds (Fig. 3). The white background reflected one-half the incident illumination and the black background 1/60. The existence of an albedo response was evident because the data obtained on the black and the white backgrounds did not overlap although the light intensity had increased 505-fold whereas the white background reflected only 30 times more light than did the black background.

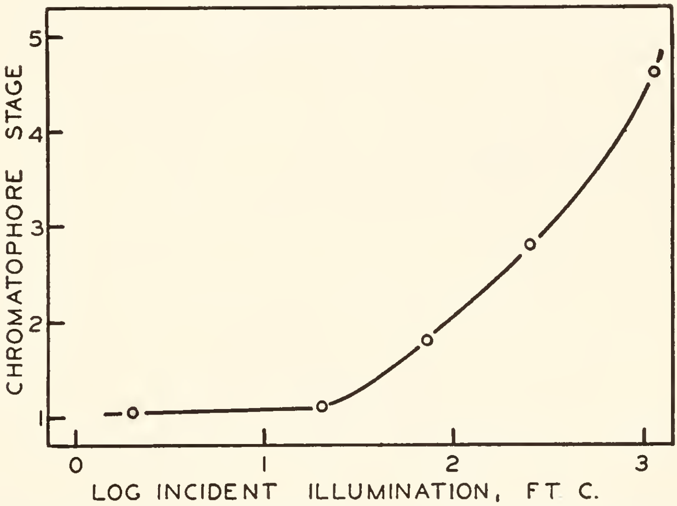

FIGURE 4. Relationship between melanophore index of eyestalkless specimens of *Sesarma reticulatum* on a white background and the logarithm of the incident light intensity.

The next experiment was designed to determine the relationship between illumination and the degree of pigment dispersion in the melanophores of eyestalkless specimens of *Sesarma reticulatum.* Eyestalkless specimens in white pans were exposed between 8:20 and 10:20 AM to the following illuminations: 2, 20, 70, 250, and 1110 ft. c. The melanin was maximally concentrated in crabs exposed to 2 ft. c. and was nearly maximally dispersed in crabs at 1110 ft. c. This experiment was repeated. Means were used in the preparation of Figure 4. The degree of pigment dispersion in eyestalkless specimens can be readily altered by changing the intensity of incident illumination. This pigment migration is presumably a direct response of the melanophores to light. The difference between the means at 2 and 1110 ft. c. was statistically significant.

Relationship between temperature and melanophore stage

The aim of this set of experiments was to determine whether the melanophores of *Sesarma reticulatum* are sensitive to temperature changes. During the daytime 10 intact crabs were placed into each of five white pans which contained sufficient sea water to cover the crabs. These crabs were then exposed to an illumination of 2 ft. c. The white background, daytime, and light intensity of 2 ft. c. were chosen because under these conditions the melanophores of the crabs were at approximately stage 3 (Fig. 2). Through use of water baths each pan was exposed for two hours to one of the following temperatures: 3.5°, 10°, 22.5°, 30°, and 36° C. The average stage of the melanophores of each crab was determined at the end of the exposure period. This experiment was performed twice.

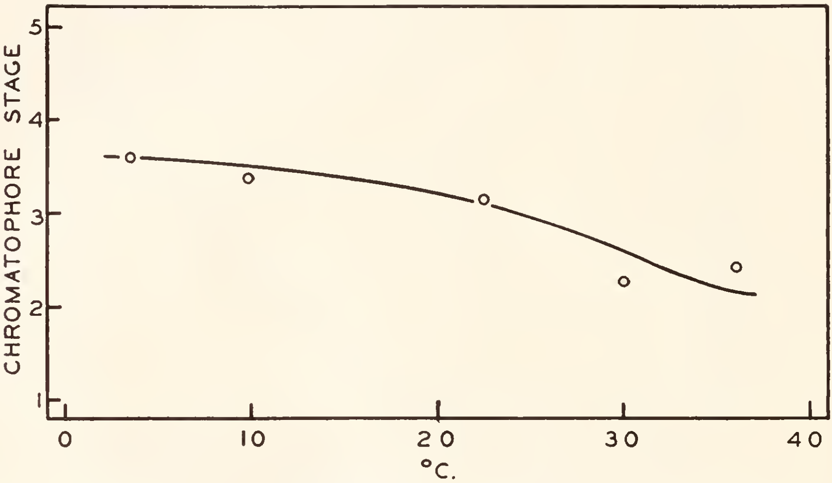

FIGURE 5. Relationship between temperature and melanophore index of intact specimens on a white background under an incident illumination of 2 ft. c. during the daytime.

An inverse relationship between temperature and chromatophore stage was apparent (Fig. 5). For purposes of statistical analysis the data obtained at (1) 3.5° and 10° C., and (2) 30° and 36° C. were grouped. The difference between the means of these two groups of data was statistically significant.

Endocrine regulation of the melanophores

The object of the final set of experiments was to determine the role of endocrines in mediating pigment migration in the melanophores of *Sesarma reticulatum*. For use in the first set of experiments of this group extracts of the optic ganglia, supraesophageal ganglia, circumesophageal connectives, and thoracic ganglia were prepared in the manner described earlier under Materials and Methods. Each extract was injected into five eyestalkless specimens in a white pan under an illumination of 2 ft. c. Under these conditions the melanin was maximally concentrated. The control consisted of eyestalkless crabs which received sea water injections. Each extract dispersed the black pigment.

This experiment was repeated twice. The data of the three experiments were averaged and the means were used in the preparation of the upper portion of Figure 6. The amount of pigment dispersion produced by each extract was highly significant.

The next experiment was similar to the previous one with the single change that the crabs were exposed to an illumination of 560 ft. c. Under this light intensity the pigment was in an intermediate degree of dispersion (see Fig. 4). Substances that concentrate as well as disperse pigment can sometimes be detected when the pigment of assay animals is in an intermediate state (Fingerman and Lowe, 1958). A pigment-dispersing substance was apparent but not a pigment-concentrating one. The experiment was repeated twice. The averaged data of the three experiments are shown in the lower portion of Figure 6. Each extract produced a statistically significant amount of pigment dispersion as was the case when extracts of these organs were injected into specimens with maximally concentrated melanin.

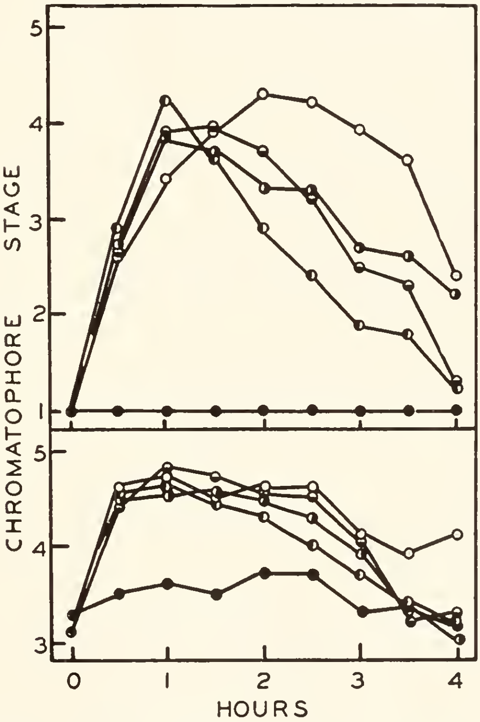

FIGURE 6. Relationships between melanophore stages and time following injection of extracts of central nervous organs into eyestalkless specimens in white containers under an illumination of 2 ft. c. (upper portion of figure) and 560 ft. c. (lower portion of figure). Optic ganglia, circles; supraesophageal ganglia, circles half-filled on bottom; circumesophageal connectives, circles half-filled on left; thoracic ganglia, circles half-filled on right; control, dots.

The remaining experiments involved assay of extracts prepared from sinus glands. Each extract was injected first into five eyestalkless crabs with maximally concentrated black pigment and pigment dispersion was noted. This experiment was repeated twice with the same results. The means were used in the preparation of the upper portion of Figure 7 where each point represents 15 crabs. The amount of pigment dispersion was statistically significant. Controls consisted of eyestalkless specimens which received sea water injections.

When an extract of sinus glands was injected into eyestalkless crabs whose black pigment was in an intermediate stage of dispersion as a result of exposure to an illumination of 560 ft. c., a transitory increase in the degree of pigment dispersion occurred. This was followed by migration of the pigment to a more concentrated level than observed in the control group. This experiment was repeated three times. Means are presented in the lower portion of Figure 7 where each point represents 20 crabs. Statistical analysis of these data revealed that the amounts of both dispersion and concentration of melanin were statistically significant.

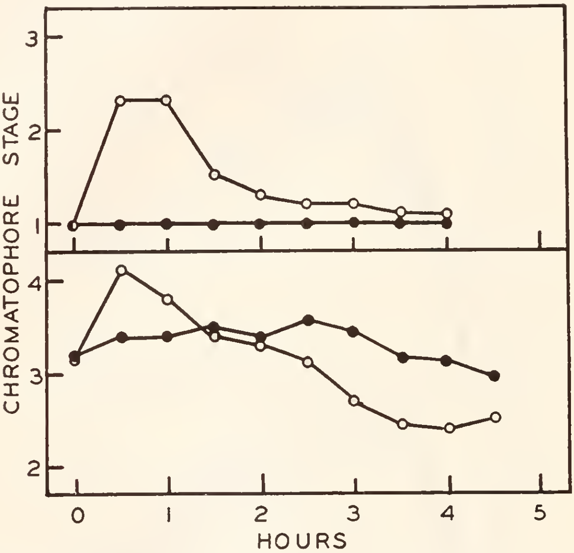

FIGURE 7. Relationships between melanophore stages and time following injection of extracts of sinus glands into eyestalkless specimens in white containers under an illuminatoin of 2 ft. c. (upper portion of figure) and 560 ft. c. (lower portion of figure). Sinus glands, circles; control, dots.

DISCUSSION

Specimens of *Sesarma reticulatum* showed a rhythm of color change which was manifested by a pale phase at night and a dark phase during the daytime (Fig. 1). Similar cycles have been observed in the fiddler crab, *Uca pugilator,* by Abramowitz (1937), in the blue crab, *Callinectes sapidus,* by Fingerman (1955), and in the swimming crab, *Macropipus vernalis* by Burgers (1959). Enami (1951) did not report on the possibility of a rhythm of color change occurring in the three species of *Sesarma* he used.

The responses to increased illumination of the melanophores in *Sesarma reticulatum, Uca pugilator* (Brown and Sandeen, 1948), and *Callinectes sapidus* (Fingerman, 1956b) are qualitatively alike. In intact specimens of these three crabs increased total illumination resulted in greater dispersion of the melanophore pigment. Enami (1951) stated that the three species of *Sesarma* he used exhibited a direct response to illumination, but he did not indicate the direction of the response. He also reported that these three species of *Sesarma* did not show a background response. However, specimens of *Uca pugilator, Callinectes sapidus,* and *Sesarma reticulatum* showed, in addition to the response to total illumination, an albedo response which called for more dispersion of melanin in crabs on a black background than on a white one. An interesting point concerning *Uca pugilator* is that in spite of the albedo response, at least during the daytime, the melanin of specimens on a white background was more dispersed than in crabs on a black background because the response to total illumination was stronger than the albedo response (Brown and Sandeen, 1948). In blue crabs, however, the albedo response was stronger than the response to total illumination (Fingerman, 1956b).

The amplitude of the response of melanophores in eyestalkless individuals of *Sesarma reticulatum* to total illumination (Fig. 4) appears to be unique among crustaceans. Maximal concentration of melanin is the typical response of brachyurans to eyestalk ablation if the crabs are not exposed to excessively high intensities of illumination (Brown, 1948). This response has been observed in a variety of crabs such as *Uca* (Carlson, 1936), *Hemigrapsus* (Bowman, 1949), and *Callinectes* (Fingerman, 1956b). As mentioned above, Enami (1951) observed three species of *Sesarma* in which the melanin dispersed maximally after eyestalk ablation instead of concentrating. Unfortunately Enami did not state the light intensity to which his crabs were exposed. At the light intensity usually recorded, about 30 ft. c., on the table tops of a laboratory, the melanin of *Sesarma reticulatum* was nearly maximally concentrated (Fig. 4), so that one would be inclined to conclude that the chromatic behavior of eyestalkless specimens of this species is more similar to that of *Uca* than to the behavior of the *Sesarma* studied by Enami. When eyestalkless *Uca* were exposed to an incident illumination as high as 3500 ft. c. (Brown and Sandeen, 1948), the black pigment dispersed only to an intermediate condition. The response of the melanophores in *Callinectes* to total illumination (Fingerman, 1956b) was not as great as was observed in *Uca* by Brown and Sandeen (1948).

In response to a rise in temperature, the black pigment of *Sesarma reticulatum* concentrated significantly (Fig. 5). This was also the response shown by the melanophores of *Callinectes* (Fingerman, 1956b). Such behavior suggested a thermoregulatory function of the black pigment. Crabs lightened at high temperatures and thereby reflected more heat from the body surface. Enami (1951) stated that the *Sesarma* he used showed a response to temperature but he did not define the nature of this response.

Extracts of central nervous organs from *Sesarma reticulatum* caused dispersion but no concentration of the pigment in melanophores (Fig. 6). However, extracts of the sinus glands caused melanin dispersion which was followed by a significant degree of melanin concentration (Fig. 7). Enami (1951) found that extracts of sinus glands and central nervous organs concentrated pigment when injected into eyestalkless specimens of *Sesarma intermedia, S. haematocheir,* and *S. dehaani.*

His experimental procedure did not allow assay for melanin-dispersing hormone, if this substance does exist in the crabs he used. The species of *Sesarma* used by Enami are not only intriguing with respect to the dispersed condition of the pigment in the melanophores of eyestalkless specimens but also with respect to the fact that the immediate effect of extracts of sinus glands from crabs investigated by Enami was concentration of pigment. Extracts of sinus glands from crabs utilized by the other investigators mentioned above always produced some dispersion of pigment.

It is interesting to note that in the assays of sinus glands of *Sesarma reticulatum* the pigment-dispersing effect preceded the pigment-concentrating one (lower portion of Figure 7). In contrast, when extracts of sinus glands from the dwarf crayfish, *Cambarellus shufeldti,* were assayed, concentration of dark red pigment occurred before dispersion (Fingerman, 1959). Red pigment in crayfishes becomes maximally dispersed after eyestalk removal (Brown, 1948). Crayfishes do not possess melanophores. Further investigation may reveal (1) the significance of this difference in behavior of the pigment-dispersing and pigment-concentrating principles in *Sesarma reticulatum* and *Cambarellus shufeldti* and (2) the reason why after eyestalk ablation the dark pigment concentrates in some crustaceans and disperses in others.

Summary and Conclusions

1. The grapsoid crab *Sesarma reticulatum* displayed a rhythm of color change which operated to disperse the pigment in the melanophores by day and concentrate it at night.

2. The crabs darkened as the intensity of illumination increased. A background response was also apparent. Melanin was more dispersed in specimens on a black background than on a white one, especially at low light intensities.

3. The degree of dispersion of the pigment in the melanophores of eyestalkless crabs was a direct function of the incident illumination. In dim light the pigment was maximally concentrated and in bright light was nearly maximally dispersed.

4. The melanin was less dispersed at high temperatures than at low. A thermoregulatory function of the melanophores was postulated.

5. Migration of melanin in *Sesarma reticulatum* is mediated by pigment-dispersing and pigment-concentrating principles.

6. The results were discussed in relation to pertinent data concerning the melanophores of crabs which have been investigated previously.

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THE CARBOHYDRATE COMPOSITION OF THE GLYCOGEN BODY OF THE CHICK EMBRYO AS REVEALED BY PAPER CHROMATOGRAPHY[1]

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The glycogen body is the term used to designate a unique mass of tissue found at the lumbo-sacral level of the avian nerve cord (Terni, 1924; Watterson, 1949). In the chick, this structure arises at 7–8 days of incubation, accumulates an appreciable amount of "glycogen" during the remainder of development and persists after hatching (Watterson, 1949; De Gennaro, 1959). The function of the glycogen body is unknown. Moreover, other questions about that structure remain unanswered at the present time. To date, there has not been any attempt to identify the carbohydrate composition of the glycogen body. It is assumed from the quantitative studies of Doyle and Watterson (1949) and Watterson *et al.* (1958) that the polysaccharide stored by that tissue is composed of glucose, on the basis of chemical tests which determine the reducing power of the hydrolysate. Such tests do not distinguish between glucose and other reducing sugars which might be present (Hawk *et al.*, 1954). It would seem important for any future work aimed at disclosing a possible metabolic function for the glycogen body to learn whether the polysaccharide stored by that structure is composed of glucose or other carbohydrates. It was felt that such information could be learned if glycogen was extracted from the glycogen body, hydrolyzed and the products of the hydrolysate separated by means of paper chromatography using known carbohydrates as controls.

MATERIALS AND METHODS

Biological and chemical

White Leghorn eggs were incubated at 38° C. and in a relative humidity of 50–60%. The lumbo-sacral regions of the spinal column of 19-day chick embryos (stage 45 of Hamburger and Hamilton, 1951) were quickly removed and put into absolute alcohol at 4° C. The vertebral column of each piece was slit open to allow rapid fixation of the glycogen body. The glycogen body was then isolated from each nerve cord after 24 hours and transferred to a 20-ml. test tube. Thirty glycogen bodies were disintegrated in 5 ml. of 30% KOH in a boiling water bath for 30 minutes. After cooling, an equal volume of absolute alcohol was added to precipitate glycogen, and the mixture was left to stand at room temperature for two hours (Hawk *et al.*, 1954). The mixture was then centrifuged for 10 minutes at 2500 rpm and the supernatant was discarded. The sediment was washed

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twice with 70% alcohol, centrifuged as before and the supernatants were discarded each time. The sediment was evaporated to dryness in the incubator at 38° C. for 10 hours.

Exactly 20 mg. of glycogen so prepared were hydrolyzed in 1 ml. of 1 N H_2SO_4 as recommended by Block *et al.* (1958) for two hours at 100° C. The amount of reducing sugar at the end of one and two hours was determined with Benedict's solution in order to ascertain that hydrolysis was complete. The hydrolysate was cooled to room temperature and passed through burets packed with 10 ml. of ion exchange resins (Amberlite, IR-4B (OH) and IRC-50 (H). Fischer Co.) to remove salts and electrolytes which are known to induce artifacts in paper chromatography (Block *et al.*, 1958). Glycogen body samples were collected by repeated washings with distilled water and then concentrated to 5 ml. by evaporation under reduced barometric pressure. The amount of reducing sugar in the sample was determined with Benedict's solution. Aqueous solutions of d-glucose, galactose, fructose, mannose and ribose, which were used as standards for chromatography, were then made in concentrations equal to that of the glycogen body so that there would be a minimum of distortion in the size and color of spots on the paper chromatogram. Final concentrations of glycogen body and of the standards used in this study ranged between 0.3–0.5 g. per 100 ml.

Paper chromatography

One drop (0.02 ml.) of each standard and glycogen body were delivered with a micropipette at equally-spaced intervals along a base line 5 cm. from the short edge of paper sheets (Whatman no. 1, 20 × 40 cm. in size). Some carbohydrate standards were also mixed together and spotted on the same paper with glycogen body to serve as controls. Each sheet was dried and fashioned into a cylinder by stapling the long edges of the paper together. The base of each cylinder was immersed in a fingerbowl containing 50 ml. of solvent. The entire apparatus was covered with a battery jar which was sealed to a glass plate to insure a saturated atmosphere. The solvent used was that recommended by Glegg and Eidinger (1954) which consisted of the following mixture: 45 ml. n-butanol, 25 ml. pyridine and 40 ml. distilled water. Aniline oxalate and resorcinol-alcohol reagents were used to develop color (Block *et al.*, 1958). Chromatograms were sprayed with those reagents after they were removed from the solvent and dried for 20 minutes at 100° F. Some chromatograms were also sprayed with ninhydrin in order to learn whether amino sugars were present (Cramer, 1955).

Unidimensional, ascending chromatograms were run at room temperature for 24 hours and then studied for the separation and positions of spots occupied by the standards and glycogen body. The multiple development method, recommended by Block *et al.* (1958) and Glegg and Eidinger (1954) for separating mixtures of simple sugars and lower polysaccharides, was also used to learn whether further separation of the glycogen body was possible. In this method, ascending chromatograms were dried and returned to the solvent every 24 hours for a total of 96 hours.

RESULTS

It was found that the position of each carbohydrate and of glycogen body was readily recognized on chromatograms after spraying with developing reagents.

Aniline oxalate stained all carbohydrates and glycogen body yellow except fructose, which gave no color, and ribose, which was pink. With resorcinol-alcohol, red spots were produced except glucose and glycogen body, which were not colored, and ribose which was stained brown. Spraying with ninhydrin gave negative results. The position of glycogen body corresponded only to that of the glucose standard in chromatograms developed after 24 and 96 hours in solvent. Effective separation of the mixtures of standards was attained after 96 hours of multiple development but did not occur with glycogen body (Fig. 1). Moreover, glucose

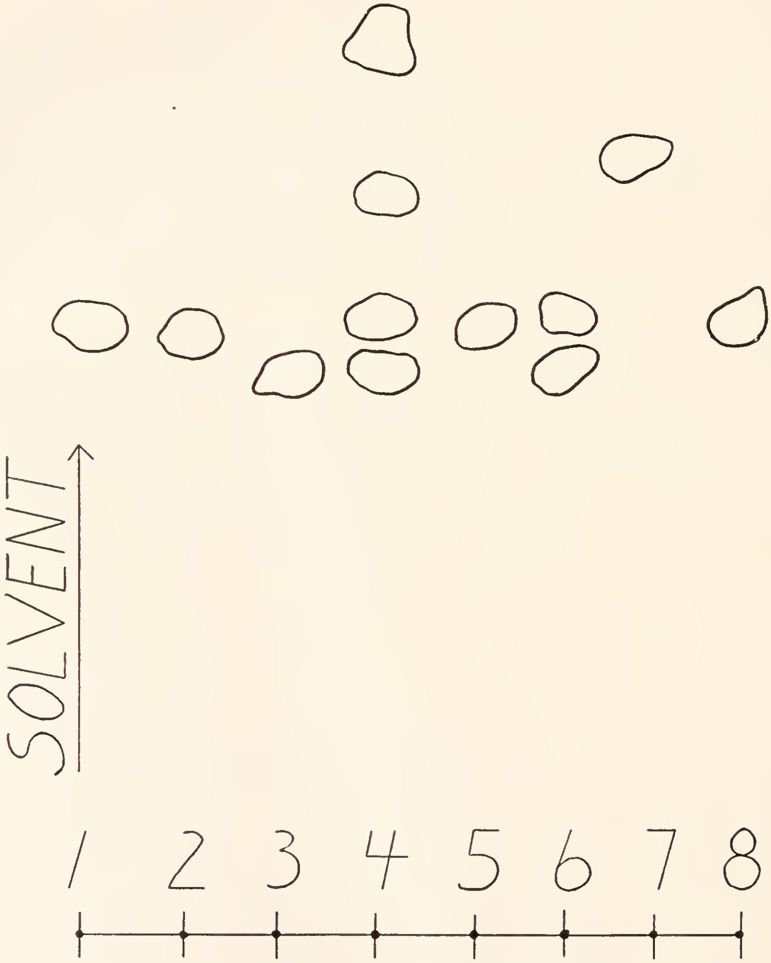

FIGURE 1. Chromatogram of glycogen body, carbohydrate standards and mixtures of standards obtained after multiple development of 96 hours. Effective separation of mixtures was attained after such treatment but did not occur with glycogen body. The position of the glycogen body corresponds only to that of the glucose standard. Explanation of numbers: 1, glycogen body; 2, glucose; 3, galactose; 4, galactose, glucose, fructose, ribose; 5, glycogen body; 6, galactose, glucose; 7, mannose; 8, glycogen body.

and galactose, which were often difficult to discern as individual spots on 24-hour chromatograms, are clearly separated from each other after 96 hours. These findings suggest that the polysaccharide stored by the glycogen body of 19-day chick embryos consists of glucose and not other carbohydrates.

Discussion

Doyle and Watterson (1949) have estimated that the glycogen body represents about 5–10% of the total glycogen of the chick embryo from 15 days of incubation to hatching. Attempts to prove that the glycogen body is a metabolic source of energy for the post-hatching period of the bird have not met with success in those experiments in which birds have been starved for various periods of time after hatching (Terni, 1924; Watterson *et al.*, 1958). Other experimentation designed to test whether the glycogen body supplies energy for morphogenesis or innervation of the posterior limb-buds has also yielded negative results (Jenkins, 1955; Watterson and Spiroff, 1949). Whether the glycogen body is metabolically inert is not known at the present time. The finding that the glycogen body consists of glucose and not other carbohydrates in this investigation might encourage a more positive view to suggest that the structure may have some importance. It would seem strange for a structure as large as the glycogen body to store vast quantities of glucose unless for some metabolic purpose. It is hoped that the results of the present study will stimulate research in that regard.

Summary

Glycogen was extracted from glycogen bodies of 19-day chick embryos with KOH and absolute alcohol, and hydrolyzed in H_2SO_4. The hydrolysate was analyzed for carbohydrates by means of paper chromatography, using known carbohydrates as controls. Special techniques are described which were employed in the preparation of the hydrolysate and for chromatography. The carbohydrate of the glycogen body was identified as glucose. Evidence is presented to suggest that the polysaccharide stored by the glycogen body of 19-day chick embryos consists of glucose and not other carbohydrates.

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CHANGES IN ABUNDANCE OF THE COMMENSAL CRABS OF CHAETOPTERUS

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The common marine annelid, *Chaetopterus variopedatus,* builds U-shaped, parchment-like tubes in the shallow waters along the Atlantic coast. The tubes are buried upright with only an inch or so of narrow siphon at either end showing above the surface of the substrate. Although the siphons are narrow, the tubes at their widest portion in the middle of the U may be as much as 4 cm. in diameter. Large tubes may reach a length of 85 cm. Of several commensal organisms that take advantage of the housing facilities set up by Chaetopterus, the most common and best known are two decapod crustaceans, *Polyonyx macrocheles* and *Pinnixa chaetopterana.* Seldom is a tube found without one or the other of these species being present. Usually adult crabs are in pairs, and especially is this true during the breeding season. A breeding pair of one species is rarely accompanied by an adult of the other species, although juveniles of the second species may often be present. The crabs are quite different; Pinnixa belongs to the family Pinnotheridae and Polyonyx to the family Porcellanidae. At the present time both species may be readily found in Chaetopterus tubes from Florida to southern New England, although this has not always been true for Polyonyx.

Enders (1905), while studying the development of Chaetopterus, noted the ratio of Polyonyx to Pinnixa at Beaufort, North Carolina. Pearse (1913) found a distinctly different ratio to exist in the region of Woods Hole, Massachusetts. These two authors appear to be the only ones who have made quantitative determinations of the relative abundance of Pinnixa and Polyonyx along the Atlantic coast. The present paper discusses the changes in relative abundance of these two crabs that have occurred in the last half century in the Beaufort and Woods Hole regions, reports their present abundance at Clearwater, Florida, and records observations on the life history of the crabs.

Grateful acknowledgment is made to Mr. and Mrs. John L. Taylor of the University of Florida, who supplied data on the crabs from Clearwater. This research was supported in part by grants from the Duke University Research Council and the National Science Foundation.

RELATIVE ABUNDANCE

Polyonyx has been called a southern crab, and it is true that its abundance on the American east coast was at one time far greater in southern than in northern waters. It was rare in the Woods Hole region at the beginning of the century.

One of the earliest records at Woods Hole is that of George M. Gray, late curator at the Marine Biological Laboratory, who found a specimen in a Chaetopterus tube in 1903. In marked contrast, about this same time, Enders (1905) found at Beaufort that approximately four-fifths of these two commensal crabs of Chaetopterus were Polyonyx. Sumner, Osburn and Cole (1911) mention Polyonyx as being present but apparently did not take any in their biological survey of the Woods Hole region. This is not surprising since Polyonyx, beyond the larval stage, is not known to occur outside Chaetopterus tubes and, with the dredging techniques used in the survey, few whole Chaetopterus tubes were obtained. However, Pearse, in 1913, found that Polyonyx constituted 22% of the crabs from 89 Chaetopterus tubes at Woods Hole. He comments on this apparent increase in abundance. Taylor, Bigelow and Graham (1957) used the increase in abundance of Polyonyx in the Woods Hole region as an example of the spread of species due to amelioration of climate. These authors, who made no determinations themselves, quote Dr. John Rankin, then naturalist at the Marine Biological Laboratory, as saying that at the present time *Pinnixa chaetopterana* "is rare in the Woods Hole region, having been replaced by the southern form, *Polyonyx macrocheles*."

TABLE I

Relative abundance of Polyonyx macrocheles *and* Pinnixa chaetopterana *in three different regions*

Region	Tubes examined	Total crabs	Polyonyx		Pinnixa	
			No.	%	No.	%
Woods Hole, Mass.						
1913 (Pearse)	89	151	33	22	118	78
1959 (Gray)	120	205	136	66	69	34
Beaufort, N. C.						
1905 (Enders)	99	174	144	83	30	17
1958–59 (Gray)	383	740	289	39	451	61
Clearwater, Fla.						
1960 (Gray)	84	141	115	82	26	18

Questioning this statement of the scarcity of Pinnixa, an examination of 120 Chaetopterus tubes was made at Woods Hole in the summer of 1959. From the 205 crabs collected, a Polyonyx-Pinnixa ratio of 66:34 was obtained. This shows that while Pinnixa cannot be considered rare in the Woods Hole region, it has to a very considerable extent been replaced over the past half century by Polyonyx. The change in relative abundance may well be due, as suggested by Taylor *et al.*, to amelioration of climate. The Chaetopterus tubes at Woods Hole in 1959 were collected from a mud substrate in Hadley Harbor, some from the intertidal zone, but most from ten to twelve feet of water. The comparative abundance of Polyonyx and Pinnixa in 1913 and 1959 is shown in Table I.

The relative abundance of these crabs at Beaufort has also changed, but in a very different way. Whereas Enders in 1905 had established a Polyonyx-Pinnixa ratio of 83:17, in 1958-59, based on 740 crabs, the ratio was found to have shifted to 39:61. Interestingly, the ratio found by Enders at Beaufort in 1905 is essentially the same as the Polyonyx-Pinnixa ratio on a sandy beach at Clearwater, Florida, in 1960 (Table I).

While amelioration of climate may be a satisfactory explanation for the increased abundance of Polyonyx in the Woods Hole region, it does not account for the increase in Pinnixa in the Beaufort area. Enders undoubtedly took his Chaetopterus from Bird Shoal, a large, continuous, sand flat covering several square miles. The western edge of this area is known as Bulkhead Shoal; the eastern portion as Guthrie Shoal. The 1958-59 populations were from three different areas: (1) a mud flat of the Newport River, a salt water estuary; (2) Bulkhead Shoal, a mixture of sand and mud; and (3) Guthrie Shoal, with a substrate of sand and shell. That the ratio of Polyonyx to Pinnixa differed in the three areas is shown in Table II. Although both species were found in tubes from all three substrates, it is evident that there is a definite correlation between the abundance of Pinnixa and the prevalence of mud. The increased abundance of Pinnixa on the more muddy substrates suggests that, for this species, at least, the crabs enter the tubes as juveniles rather than as planktonic larvae. Pinnixa is a mud crab. Although more commonly found there than elsewhere, it is by no means limited to Chaetopterus tubes. It is frequently found in the mud tubes of the annelid, *Amphitrite ornata*, and occasionally in tubes of other dwellers of mud and sand flats. It is not uncommon on the shoals behind the Outer Banks, near Cape Hatteras, North Carolina, although the tubes of Chaetopterus are rare in this locality. On the other hand, Polyonyx has been reported from Chaetopterus tubes only.

TABLE II

Correlation between abundance of Polyonyx and Pinnixa and type of substrate at Beaufort

Substrate	Tubes examined	Total crabs	Polyonyx		Pinnixa	
			No.	%	No.	%
Sand-shell (Guthrie Shoal)	139	261	141	54	120	46
Sand-mud (Bulkhead Shoal)	133	274	94	34	180	66
Mud (Newport River)	111	205	54	26	151	74

LIFE-HISTORY AND ECOLOGY

Statements to the effect that neither of these crabs, when adult, can escape from the tubes which they entered as larvae or juveniles need qualification. While it may be true that adult crabs, especially Pinnixa, cannot escape through the openings of young Chaetopterus tubes with narrow chimneys, it is not true of crabs in older tubes with wide chimneys. In the field Pinnixa has been observed on several occasions leaving chimneys of wide diameter when the tubes were disturbed in the process of removal from the substrate. Under experimental conditions in the laboratory crabs enter and leave the tubes. If the diameter of the chimney is too narrow the crab bites a hole in the parchment at the base of the chimney and makes his entrance or exit at this point. This behavior, rare with Polyonyx, has been observed often with Pinnixa. Pearse (1913) noticed that Pinnixa placed in aquaria with Chaetopterus were able to find their way into the tube, although he did not observe how this was accomplished. Probably under natural normal conditions neither species would have reason for leaving the tubes.

In September and October, when a new generation of Chaetopterus is developing and the tubes are new and being expanded rapidly, only small crabs are to be found in small tubes. It is assumed that the young crabs grow with the Chaetopterus tubes and are not replaced later by other crabs. It is not known if the crabs live more than one year. Judging by the length of the breeding season and the sizes of the crabs carrying eggs, it is thought that each species may breed more than once during the summer, at least in the more southern part of the range. In the Beaufort region the egg-carrying season for Pinnixa ends in early September, but Polyonyx continues to carry eggs in December. On November 30, 1959, 80% of the adult female Polyonyx taken had eggs, but a month later, December 29, none were with eggs. Enders (1905) reported that Polyonyx in the Beaufort area was breeding on October 25, the date of his last field observation. Egg-laying for both Pinnixa and Polyonyx in the Beaufort region is resumed in April. An examination of crabs from three different habitats on March 28-30, 1960, showed none with eggs, but several adult female Polyonyx appeared ready to molt. Presumably molting precedes egg-laying. On April 22 all females of both species were carrying eggs in early stages of development. It is possible that this is a late date for egg-laying, since March, 1960, was an abnormally cold month, with water temperatures in Beaufort Harbor dropping to freezing on several occasions. Female crabs of both species taken February 20 at Clearwater, Florida, were carrying eggs. When egg-laying begins or ends in the Woods Hole region was not determined.

TABLE III

Carapace width of mature Polyonyx and Pinnixa from three different regions. The minimum among females is for egg-bearing individuals. Measurements are in millimeters.

	Polyonyx				Pinnixa			
	No.	Max.	Min.	Ave.	No.	Max.	Min.	Ave.
Woods Hole, Mass.								
♂	67	11.3	8.0	9.9	32	15.1	7.3	12.3
♀	70	14.4	10.1	12.8	35	17.4	9.4	13.4
Beaufort, N. C.								
♂	143	11.1	7.1	9.2	220	15.0	6.9	12.0
♀	141	16.0	10.1	13.0	221	17.5	8.4	13.5
Clearwater, Fla.								
♂	55	9.2	6.4	7.6	12	12.0	8.5	10.5
♀	60	12.9	8.4	10.5	14	14.0	8.9	11.8

In the summer months, while females are carrying eggs, there is usually one pair of either Pinnixa or Polyonyx, but not both species, present in any particular tube. If another species is present it is usually a small immature crab. However, beginning in mid-August in the Beaufort region, as new juvenile crabs appear, it is possible to find several young crabs, of either species, in the same tube with adults. In

August the majority of juveniles are Pinnixa. Forty-one of 49 juveniles from 100 Chaetopterus tubes, August 16-26, 1960, were Pinnixa. During the fall months the number of juveniles in Chaetopterus tubes, especially of Polyonyx, increases.

In both species females are larger than males. The carapace widths of crabs from Beaufort and Woods Hole are essentially the same (Table III). Crabs from Clearwater, Florida, averaged somewhat smaller. This is perhaps because the Florida specimens were taken in February, while the Beaufort and Woods Hole material was collected in July and August. In the Beaufort region it is thought that both Pinnixa and Polyonyx males are mature when they reach a carapace width of approximately 7 mm. The smallest females of Pinnixa with eggs measured 8.4 mm. and the smallest Polyonyx females with eggs 10.1 mm. However, Florida female Polyonyx as small as 8.4 mm. had eggs. It is not known if Pinnixa and Polyonyx taken in Florida in comparable numbers and in comparable seasons are in reality smaller than those from Beaufort and Woods Hole.

Living in protective Chaetopterus tubes the crabs are not subject to the same degree of predation or to the same fluctuations in environmental conditions that more exposed animals are. This is particularly true of those in the intertidal zone. Both Polyonyx and Pinnixa seek positions at the posterior end of the worm and are thus near one siphon or the other. In fall and winter months the siphons of Chaetopterus tubes are frequently cut off by the blue crab, *Callinectes sapidus*, and by birds, and undoubtedly many commensal crabs are then caught and eaten by predators. Temperatures within the tubes are more stable than on the sand flats. On a hot August day at Beaufort, when the temperature of water in small tide pools left at low tide registered 35° C., the maximum temperature within twelve Chaetopterus tubes tested was only 29°.

That Chaetopterus holds no strong chemical attraction for adult crabs has been demonstrated many times by students in the laboratory, but whether there is a chemical attraction for young crabs is not known. Experiments using the technique of Davenport (1950) in which a crab placed in a Y-tube has a choice of going upstream toward a jar containing Chaetopterus or toward a jar of sea water have produced negative results. Eighty per cent of the crabs failed to go in either direction. It seems probable that, at least for Pinnixa, the young crabs find their way by chance to the Chaetopterus tubes.

Of crabs other than Pinnixa and Polyonyx that invade Chaetopterus tubes in the Beaufort area, the mussel crab, *Pinnotheres maculata* is most often found. For the most part these are young males. The oyster crab, *P. ostreum*, occasionally with eggs, has been taken also. Rarely, the small spider crab, *Pelia mutica*, may be found. Usually these are in tubes bearing Pinnixa, seldom with Polyonyx. Pinnixa is not as clean a crab as Polyonyx. It commonly is covered with the bryozoan, *Triticella elongata*, and occasionally has young sea squirts, *Styela plicata*, attached to its legs or back. A hydroid, *Clava* sp., may also be found on Pinnixa. These sessile invaders are always attached to the crab, never to the wall of the Chaetopterus tube.

DISCUSSION

It is difficult to offer a satisfactory explanation for the changes in relative abundance of the two commensal crabs, Pinnixa and Polyonyx. Amelioration of climate, suggested by Taylor *et al.*, seems a satisfactory explanation for the change in distribution in the Woods Hole region. Here it is assumed that the shift in relative numbers has been a gradual one over a long period of time. However, the same explanation does not suffice for the shift in the opposite direction at Beaufort, where Polyonyx shows a marked decrease in relative numbers during the same period of time. At Beaufort two possibilities present themselves. There is no evidence one way or the other as to whether the change was gradual or sudden. If substrate is of importance, it is quite possible that Bird Shoal, where supposedly Enders secured his Chaetopterus, has changed materially in the past fifty years and perhaps has more mud mixed with sand at present than formerly. Considering the amount of dredging of the channels in recent years this seems entirely possible. In fact, dredging in the fall of 1959 added several inches of mud to the substrate on the inner side of Bird Shoal. The more muddy substrate could create an advantage for Pinnixa, which is quite at home in mud.

Another suggestion is that the change may have been sudden, following the great depletion of Chaetopterus as a result of a series of hurricanes in 1954 and 1955, which so extensively washed Chaetopterus tubes out of the substrate that they were scarce in the area in 1956 and only moderately plentiful in 1957. Chaetopterus did not again appear in abundance until 1958. The three hurricanes of 1955 between August 11 and September 15 were particularly disastrous to Chaetopterus tubes. Coming at a time when many Pinnixa had finished breeding and when the abundance of juveniles was far greater for Pinnixa than for Polyonyx, the former species would have been favored as the one to populate new Chaetopterus tubes that might be forming. Large Chaetopterus tubes were very scarce following these hurricanes. Pinnixa, in contrast to Polyonyx, can rapidly burrow into the substrate. In the laboratory Pinnixa has been observed to disappear into the substrate almost immediately, while Polyonyx remained on the surface even after several days. If separated from Chaetopterus tubes, Pinnixa would therefore have a much greater chance for survival than would Polyonyx, which is not known to occur in the adult stage outside of Chaetopterus tubes.

It is not clear when the crabs enter the tubes, whether as planktonic larvae, as juvenile crabs, or both. Since young Chaetopterus tubes have only young crabs in them it is assumed that the crabs grow with the tubes. In the case of Pinnixa it is thought that the tubes are entered by juveniles, since the crabs are able to live outside of Chaetopterus tubes.

It is obvious that Pinnixa, although it seems to prefer Chaetopterus tubes to other environments, is not an obligate commensal of Chaetopterus; nor are the bryozoans, hydroids and sea squirts that are so frequently attached to Pinnixa obligates of the latter. On the other hand, however, Polyonyx, beyond the planktonic larval stage found only in Chaetopterus tubes, must be considered an obligate commensal of Chaetopterus. Other than obtaining food brought in by the host, the chief benefit appears to be protection. Polyonyx does not burrow and is thus at a disadvantage when liberated among predators on a sandy substrate.

SUMMARY

1. The relative abundance of two species of commensal crabs of Chaetopterus, *Pinnixa chaetopterana* and *Polyonyx macrocheles,* has been determined in the

regions of Clearwater, Florida; Beaufort, North Carolina; and Woods Hole, Massachusetts. The marked changes in relative abundance of the two crabs that have occurred during the last half century at Beaufort and Woods Hole are discussed.

2. Comparative observations on the life-history and ecology of the two crabs are recorded.

3. Pinnixa is considered a facultative commensal and Polyonyx an obligate commensal of Chaetopterus.

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INTERACTIONS BETWEEN ARBACIA SPERM AND S^{35}-LABELLED FERTILIZIN[1]

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The sperm of *Arbacia punctulata* and other echinoids agglutinate reversibly when mixed with fertilizin of the same species. Fertilizin is the sole large-molecular constituent so far detected in the jelly coat which surrounds the sea urchin egg, and consequently any reaction between this substance and sperm is of interest in studies of fertilization. Although there is general familiarity with the basic facts of the agglutination reaction, many aspects require clarification. For instance, the molecular events accompanying reversal are quite obscure. Attempts to understand these reactions have been advanced by the use of serological models (Lillie, 1914; Tyler, 1948, 1959). These models have led to the view that fertilizin is a large multivalent molecule which combines with two or more sperm per molecule to bring about agglutination. An antigen-antibody type union is thought to form between sites on fertilizin and complementary sites on the sperm surface associated with a protein termed antifertilizin. Reversal of agglutination occurs, according to this view, when fertilizin is altered to a univalent state, *i.e.*, the multivalent molecule is fragmented into components with no more than one reactive site on each part. Phenomena analogous to this explanation of sperm reversal are found in demonstrations of univalent antibodies in nature (Heidelberger, *et al.*, 1940), and in the experimental conversion of multivalent antibodies to a univalent form (Tyler, 1945; Porter, 1959). Multivalent fertilizin has been converted to a univalent form by various agents: ultraviolet and x-irradiation, proteolytic enzymes, hydrogen peroxide, and heat (Tyler, 1941; Metz, 1942, 1954).

The idea that sperm reversal results from conversion of fertilizin from a multivalent to a univalent form implies that the agent(s) of this conversion is sperm or some component(s) of sperm. This has led to questions regarding the mechanisms of such conversion and the molecular nature of the product. As an approach to these problems the present studies, employing S^{35}-labelled fertilizin (Tyler and Hathaway, 1958), were undertaken. The high sulphate content of most fertilizins (Vasseur, 1947, 1948; Tyler, 1959), and the possible role of such sulphate in fertilizin agglutination of sperm (Vasseur and Carlsen, 1948; Vasseur, 1952), suggested the experiments described in this paper. These show that sperm bind, and subsequently release, fertilizin S^{35}. This uptake and release of S^{35} label is associated with fertilizin agglutination of sperm and its reversal. These results, and

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other preliminary observations, have been published earlier in abstract form (Hathaway and Tyler, 1958; Hathaway, 1959).

MATERIALS AND METHODS

Arbacia punctulata for all experiments were provided by the Marine Biological Laboratory Supply Department.

S^{35}-labelled fertilizin was produced by the procedure of Tyler and Hathaway (1958). This involved the injection of a sea water solution of $Na_2S^{35}O_4$ into females previously shed of gametes. Three injections of 50 microcuries each were administered on alternate days, during which time the animals were maintained in aerated sea water abundantly supplied with algal food. During the six days of injection treatment the animals apparently incorporated the inorganic S^{35} into newly produced eggs. At the end of this time the animals were left overnight in running sea water, and were then caused to shed their eggs by means of KCl injection. After repeated washings in cold sea water, the eggs were subjected to a pH of 3.5 to 4.0, which dissolved the fertilizin from them. Such fertilizin preparations, after dialysis, were found to be highly radioactive and to give the usual reversible agglutination reaction with sperm. Aliquots of a single fertilizin preparation were utilized in all of the experiments described below.

Sperm were collected by electrical stimulation of exposed gonads. Routine washing of sperm consisted of suspending about 3 ml. semen in 12 ml. cold sea water. After centrifugation, the sperm were re-suspended in sea water to a final volume of 5 times the volume of packed sperm. Radioactivity was measured in a windowless gas-flow counter. The "soft" beta emission of S^{35} is masked by sperm; therefore radioactive assays in these experiments were always made on supernatant fluids only.

RESULTS

S^{35} uptake by sperm

Ten experiments were performed to test the capacity of sperm to remove radioactivity from solutions of S^{35}-fertilizin. In these, 1 ml. each of sperm suspension and labelled fertilizin were mixed at 0° C. The mixtures were centrifuged 30 seconds later and a 0.5-ml. sample of the supernatant (No. 1) was removed for S^{35} assay. The results of the ten experiments are given in Table I (Columns A, B, C, and D). The S^{35} activity of a 0.5-ml. sample of control fertilizin solution (1 ml. fertilizin plus 1 ml. sea water) is given in Column A. Column B gives the activity of corresponding supernatants of fertilizin-sperm mixtures (1 ml. fertilizin plus 1 ml. ca. 20% sperm) obtained by centrifuging immediately after mixing. Column C incorporates an adjustment (see footnote below Table I). Finally, as seen in Table I, Column D, the supernatant of such sperm-fertilizin mixtures contained an average of 28.0 ± 1.1% of the initial radioactivity. Evidently the sperm bound 72% of the S^{35} label initially present in the fertilizin solution. The 28% of unbound fertilizin could represent excess fertilizin or labelled non-fertilizin contaminant.

Release of bound S^{35} label from sperm

Since preliminary experiments (Hathaway and Tyler, 1958) had indicated a release of bound S^{35} from sperm, further tests were performed. These utilized the material remaining from the above ten experiments. Sperm were re-suspended in remaining supernatant and left 45 minutes at room temperature. These suspensions were then centrifuged, and the supernatant (No. 2) was assayed for S^{35} (Table I, Columns E and F). As seen in Column G, these supernatants contained an average of 72.7 ± 2.5% of the S^{35} in the system. This striking increase in supernatant S^{35} concentration is evidently a result of the release of bound S^{35} from sperm.

TABLE I

S^{35}[*] *in supernatants of sperm and labelled fertilizin mixtures*

Experiment number	A	B	C	D	E	F	G
	Control unabsorbed fertilizin cpm/0.5 ml.	Supernatant #1 actual cpm/0.5 ml.	Supernatant #1 adjusted[**] cpm/0.5 ml.	% total counts recovered in supernatant	Supernatant #2 actual cpm/0.5 ml.	Supernatant #2 adjusted[‡] cpm/0.5 ml.	% total counts recovered in supernatant
1	308	87	78	25	374	263	85
2	308	98	88	29	333	238	77
3	335	119	107	32	348	253	76
4	335	108	97	29	338	244	73
5	356	123	111	31	303	224	67
6	356	104	94	26	257	191	53
7	311	72	65	21	333	233	75
8	311	84	76	24	318	226	73
9	311	107	96	34	302	221	71
10	311	91	82	29	336	239	77
Average				28.0 ± 1.1			72.7 ± 2.5

[*] Counts per minute (cpm).

[**] Adjusted to equal 25% of supernatant by allowing for relative volume of packed sperm: actual count × 0.90 (sample was 0.5 ml. out of a total of 2.0 ml., of which 0.2 ml. was packed sperm).

[‡] Adjusted to equal 25% of supernatant and to allow for relative volume of packed sperm (Actual count × $\frac{1.3}{4}$ ÷ 0.5, since sample was 0.5 ml. of total volume of 1.5 ml., of which 0.2 ml. was packed sperm), and for S^{35} removed in first sampling $\left(+ \frac{\text{Actual count first sample} \times 0.90}{4} \right)$.

Absorption of S^{35} by pretreated sperm

Earlier experiments using sperm agglutination as the test for fertilizin have established that sperm fail to absorb agglutinin following treatment with excess fertilizin. It seemed of interest, then, to determine if sperm behave similarly with regard to S^{35} label.

Accordingly, a test was performed to see if sperm can bind S^{35} during a second mixing with labelled fertilizin. The experiment had four steps: (A) Sperm and fertilizin were mixed and immediately centrifuged, and the supernatant was assayed for S^{35} (Table II, Column A). (B) Sperm were re-suspended in supernatant for 45 minutes, re-centrifuged, and the supernatant again assayed (Column B). (C) Remaining supernatant was removed. Sperm were re-suspended in a fresh labelled fertilizin solution, immediately re-centrifuged, and the supernatant was assayed (Column C). (D) Sperm were re-suspended in the remaining supernatant for 45 minutes, re-centrifuged, and the supernatant was again assayed (Column D). Results show that the sperm in steps A and B absorbed and released S^{35} as expected, but that in steps C and D, following the initial fertilizin treatment, the sperm bound practically no S^{35}. Control sperm (treated with sea water rather than fertilizin in steps A and B) absorbed all but 36% of the label in step C (Table II, Column C). These results show that if fertilizin is included in steps A and B it causes alterations in sperm that greatly reduce their capacity to bind S^{35} in step C.

Table II

Recovery of S^{35} in supernatants of sperm treated twice with labelled fertilizin

	A Experimental	B Experimental	C		D	
			Experimental	Control	Experimental	Control
Actual counts/min./0.5 ml. of supernatant	84	318	339	121	378	213
Adjusted counts/min./ 0.5 ml. of supernatant	76*	226**	305*	109*	322**	166**
% total counts‡ recovered in supernatant	24	73	98	36	103	53

Columns represent successive supernatants. A, B, C, D, as described in text. Data in columns A and B appear as experiment 8 in Table I.

Control sperm were treated with sea water instead of fertilizin in steps A and B.

* Adjusted to equal 25% of supernatant by allowing for relative volume of packed sperm: Actual count × 0.90 (sample was 0.5 ml. out of a total of 2.0 ml., of which 0.2 ml. was packed sperm).

** Adjusted to equal 25% of supernatant and to allow for relative volume of packed sperm (actual count $\times \frac{1.3}{4} \div 0.5$, since sample was 0.5 ml. of total volume of 1.5 ml., of which 0.2 ml. was packed sperm), and for S^{35} removed in first sampling $\left(+\frac{\text{Actual count first sample} \times 0.9}{4}\right)$.

‡ There were 311 counts/min./0.5 ml. of unabsorbed fertilizin solutions. This value is used as a baseline from which calculations were made of % total counts recovered in supernatants.

Control sperm in step D did not release as much S^{35} as was expected on the basis of the previous experiments (*cf.* Table I). It has been shown previously (Hathaway and Tyler, 1958) that excessive sperm washing impairs the capacity of the sperm for subsequent release of bound materials. Possibly the centrifuging and stirring of control sperm in steps A–C similarly explain the reduced release of S^{35} in step D. It is interesting in this connection that Rybak (1955) observed that sea urchin sperm washed and centrifuged two times in sea water fail to reverse after agglutination with fertilizin.

Failure of released S^{35} to bind to fresh sperm

Experiments outlined in a previous section show that a large proportion of S^{35} label initially bound to sperm is subsequently released. Consequently an experiment was performed to examine the extent to which previously bound and released S^{35} can be taken up by fresh sperm. (A) Sperm and S^{35} fertilizin were mixed, immediately centrifuged, and the supernatant was assayed (Table III, Column A).

(B) Sperm were re-suspended for 45 minutes, re-centrifuged, and the supernatant was again assayed (Column B). (C) The supernatant was decanted and mixed with fresh sperm, centrifuged immediately, and the supernatant was assayed (Column C). (D) Sperm were re-suspended for 45 minutes, re-centrifuged, and the supernatant was assayed again (Column D). The results show that S^{35} was absorbed and released by sperm in steps A and B, as expected. However, in step C the fresh sperm absorbed only 10% of the S^{35} from the previously absorbed solution. The 10% absorbed may represent excess fertilizin that was not absorbed in the initial treatment (Column A). In any event, the sperm failed to absorb 90% of the S^{35} label in this second absorption, whereas the sperm in the first absorption took up all but 24% of the S^{35} activity. These results are most readily explained by assuming that the S^{35} activity taken up and subsequently released in the first absorption is not re-absorbed by the second treatment with sperm. Column D shows that there is a little change in the amount absorbed by the second sperm treatment even after 45 minutes.

TABLE III

S^{35} in supernatants reacted twice with fresh sperm

	A	B	C	D
Actual counts/min./0.5 ml. supernatant	81	298	154	152
Adjusted counts/min./0.5 ml. supernatant	73*	212**	139*	134**
% total counts recovered in supernatant	24	70	90	88

Columns represent successive supernatants A, B, C, D, as described in text.

In steps A and B unabsorbed fertilizin solution had 304 cpm/0.5 ml. In steps C and D, the fertilizin solution before absorption had 152 cpm/0.5 ml.

* Adjusted to equal 25% of supernatant by allowing for relative volume of packed sperm: Actual count × 0.90 (sample was 0.5 ml. out of a total of 2.0 ml., of which 0.2 ml. was packed sperm).

** Adjusted to equal 25% of supernatant and to allow for relative volume of packed sperm (Actual count $\times \frac{1.3}{4} \div 0.5$, since sample was 0.5 ml. of total volume of 1.5 ml., of which 0.2 ml. was packed sperm), and for S^{35} removed in first sampling $\left(+\frac{\text{Actual count first sample} \times 0.9}{4}\right)$.

Non-diffusibility of S^{35}-labelled substances

Supernatants from experiments 1, 2, and 3 (Table I) were dialyzed to determine if the S^{35} material would pass through cellophane. No S^{35} diffused through the membranes in any of the tests. These results probably mean that the S^{35} in supernatants is associated with a high molecular weight substance. Alterations of fertilizin by whole sperm apparently do not involve a release of free sulphate.

Absorption of S^{35} label by heterologous sperm

Experiments were performed to see if sperm of other species can bind S^{35}-labelled *Arbacia* fertilizin. Sperm of *Chaetopterus variopedatus, Placopecten* *magellanicus*, and *Spisula solidissima* were used. As in other experiments, sperm and fertilizin were mixed and centrifuged immediately, and the supernatant was assayed for S^{35} (Table IV, Columns B and C). *Arbacia* sperm absorbed 68% of the fertilizin as expected. *Placopecten* and *Spisula* sperm also absorbed substantial amounts of activity (97% and 51%, respectively). *Chaetopterus*, on the contrary, failed to absorb S^{35} activity from the *Arbacia* fertilizin during the 30-second exposure.

To test for release of bound *Arbacia* fertilizin the sperm were re-suspended in their supernatants and again centrifuged after a 45-minute interval. As seen in Table IV, Columns D and E, *Arbacia* sperm released appreciable amounts of S^{35}. *Spisula* and *Placopecten*, however, failed to release activity. Furthermore, *Chaetopterus* sperm absorbed 94% of the S^{35} activity in this part of the experiment. Evidently *Chaetopterus* sperm absorbed fertilizin quite slowly or did so only after damage following a preliminary centrifugation (Compare Columns C and E, Table IV). The results show that the heterologous sperm irreversibly bind the S^{35}-labelled *Arbacia* fertilizin.

TABLE IV

S^{35} *in supernatant of mixtures of Arbacia fertilizin and heterologous sperm*

Fertilizin mixed with the sperm of:	A Unabsorbed fertilizin cpm/0.5 ml.	Supernatant No. 1		Supernatant No. 2	
		B Adjusted** cpm/0.5 ml.	C % total radio-activity remaining in solution	D Adjusted‡ cpm/0.5 ml.	E % total radio-activity remaining in solution
_Arbacia_*	335	107	32	253	76
Spisula	335	166	49	64	19
Placopecten	356	12	3	11	3
Chaetopterus	311	310	100	6	6

* These data appear as experiment 3 in Table I.

** Adjusted to equal 25% of supernatant by allowing for relative volume of packed sperm: actual count × 0.90 (sample was 0.5 ml. out of a total of 2.0 ml., of which 0.2 ml. was packed sperm).

‡ Adjusted to equal 25% of supernatant and to allow for relative volume of packed sperm (actual count $\times \frac{1.3}{4} \div 0.5$, since sample was 0.5 ml. of total volume of 1.5 ml., of which 0.2 ml. was packed sperm), and for S^{35} removed in first sampling $\left(+\frac{\text{Actual count first sample} \times 0.90}{4}\right)$.

Whether the S^{35} label is bound to the sperm surface or to the subsurface material remains to be established. The results with *Chaetopterus* suggest that damage by centrifugation is required. Such damage may also be a factor in absorption of the S^{35} label by the other heterologous species. In any event, none of the heterologous sperm gave an agglutination reaction when mixed with *Arbacia* fertilizin.

DISCUSSION

The use of S^{35}-labelled fertilizin permits a recognition of two separable steps in the complex reaction between fertilizin and sperm. These steps are (1) an immediate absorption of fertilizin to sperm, and (2) a subsequent release of a large fraction of the absorbed materials. The occurrence of these two successive reactions is consistent with the visually observed agglutination and reversal of sperm by fertilizin. The S^{35} data are consistent, also, with previous work.

Absorption of S^{35} fertilizin

Absorption of fertilizin by sperm has been indicated by several methods. Sperm decrease the agglutinating activity of fertilizin solutions (Lillie, 1913; Tyler, 1941; Metz, 1945). Since the agglutinating activity is a property of fertilizin, these experiments suggest fertilizin absorption by sperm. Work with *A. lixula* (Monroy *et al.,* 1954) shows that sperm remove the carbohydrate constituent, fucose, from fertilizin solutions. The present studies show that sperm absorb sulphur constituents from fertilizin solutions. There seems to be little doubt that an immediate absorption of fertilizin to sperm is a phenomenon of general occurrence in echinoids.

Release of S^{35} fertilizin

The results of this study show that after the immediate absorption of S^{35} fertilizin there is a subsequent release of a large fraction of the S^{35}. This result suggests an enzymatic degradation of the absorbed fertilizin, in which parts of fertilizin molecules are released to the medium. There have been many reports which infer the existence of a fertilizin-splitting enzyme on sperm. The viscosity of fertilizin solutions decreases when sperm or sperm extracts are added (Vasseur, 1951; Monroy and Tosi, 1952; Lundblad and Monroy, 1950). A later reevaluation of this work, however, led to the conclusion that viscosity decreases were due to absorption of fertilizin rather than to its enzymatic degradation (Monroy *et al.,* 1954). Other investigators have inferred the existence of fertilizin-splitting enzymes from examination of the effects of sperm extracts on the jelly coats of eggs (Hartmann *et al.,* 1940a, 1940b; Monroy and Ruffo, 1947; Numanoi, 1953). Although sperm extracts can be prepared which cause the disappearance of egg jellies, conclusions based on this observation must take into account the fact that sperm extracts can precipitate and contract the jellies rather than dissolve them. The necessity for distinguishing between such precipitating agents (antifertilizin) and other agents which might lyse the jelly coat and degrade fertilizin has been repeatedly stated (Tyler and O'Melveny, 1941; Krauss, 1950; Monroy and Tosi, 1952; Monroy *et al.,* 1954; Brookbank, 1958).

Experiments have been designed to distinguish between antifertilizin and suspected jelly-dispersing agents. Ishida (1954) observed that sperm treated with fertilizin still caused the disappearance of the egg jelly coat. Presumably the antifertilizin of these sperm was saturated with fertilizin so that the effects on the jelly coat were not due to antifertilizin. Brookbank (1958) has demonstrated a clear distinction between antifertilizin and an egg jelly-dispersing agent in *Mellita* sperm extracts. The jelly-dispersing agent is inactivated by acid (pH 4.0) and heat (70° C. for two minutes). Antifertilizin is not affected by heat or acid in the ranges used. The *Mellita* jelly-dispersing agent does not affect the sperm agglutinating titer of fertilizin in solution. The effects of this agent are limited to dispersing the jelly coat.

Recent experiments with *Arbacia punctulata* have indicated an egg jelly-dispersing agent from sperm. This agent is heat- and acid-labile (80° C. for 5 minutes, pH 3.0), and inactive at reduced temperatures (Hathaway, Warren and Flaks, 1960).

In the present experiments, whole sperm have been observed to divide fertilizin solutions into two fractions. One fraction appears to be bound permanently to sperm. The other fraction is only transiently associated with sperm. If the sperm are causing a fragmentation of fertilizin molecules, it might be expected that some parts of the molecule are freed from the sperm. The relative amounts of bound and freed S^{35} were roughly equal. The dialysis experiments support the idea that released S^{35} is associated with a large molecular type. Although these experiments do not conclusively show an enzymatic breakdown of fertilizin by sperm, the results are consistent with the hypothesis that an active fertilizin depolymerase is present in sperm.

A further possible explanation of S^{35} release by sperm is that some fertilizin molecules may be permanently adsorbed to sperm, while other fertilizin molecules combine with sperm receptors which themselves separate from the sperm. The complex of fertilizin and separated sperm receptors would then be recovered in supernatants. This fertilizin would have its reactive sites occupied by sperm receptors and would not react with additional sperm. The release of sperm receptors during reversal of agglutination was first suggested by Lillie (1914). It is known that sperm substances, presumably from the cell surface, are "soluble" enough to be detected in the medium (Monroy *et al.*, 1954; Köhler and Metz, 1960). It has also been demonstrated that fertilizin solutions cause the release of sialic acid-containing constituents from sperm (Warren, Hathaway and Flaks, 1960). Further experiments are required to decide if either of the suggested mechanisms is operative in the reaction of sperm and fertilizin.

Sperm agglutination and fertilization

The uptake and release of fertilizin S^{35} by sperm presents a partial explanation of the molecular events accompanying sperm agglutination and reversal. Agglutination evidently results from the binding of fertilizin to sperm. Reversal appears to be accompanied by the release of some S^{35}-containing fertilizin components. The release of S^{35} substance is evidence that reversal is caused by a breakdown of fertilizin.

There is an obvious value in a mechanism which would permit the egg to "trap" an approaching sperm. The immediate absorption of dissolved S^{35} fertilizin to sperm suggests that similar bonds hold the sperm trapped in the jelly coat of the egg. Once the sperm is trapped, however, it must proceed through the jelly coat to the egg membrane. Such a process would be facilitated by a sperm agent which would cause the breakdown of fertilizin. The agent which causes S^{35} release might serve this purpose in the case of fertilization in sea urchins.

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SUMMARY

1. Reactions between *Arbacia* fertilizin and sperm were studied with preparations of S^{35}-labelled fertilizin. Experiments consisted of mixing fertilizin and sperm, centrifuging the sperm from the suspension, and assaying the supernatant for radioactivity. In this way the absorption of fertilizin to sperm could be measured as a loss of radioactivity from the supernatant.

2. Results of 10 experiments show that there was an initial absorption by sperm of all but 28% of the fertilizin. This was rapidly followed by a release of more than one-half the absorbed material.

3. Sperm which have reacted with fertilizin do not absorb additional fertilizin upon further treatment with this substance.

4. Fertilizin solutions which have reacted with sperm are not appreciably absorbed by fresh sperm.

5. Dialysis shows that the S^{35}-labelled material absorbed to and released from sperm is non-diffusible.

6. Heterologous sperm from three species absorb *Arbacia* fertilizin but do not release it.

7. The parallel between absorption and release of S^{35} by sperm, and the agglutination and reversal of sperm by fertilizin, is noted, and it is concluded that the behavior of S^{35}-labelled material in these experiments is a molecular expression of the reversible agglutination reaction.

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RESPIRATION RATES IN PLANARIANS. II. THE EFFECT OF GOITROGENS ON OXYGEN CONSUMPTION[1]

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A number of drugs, known as goitrogens, interfere with one or the other of the mechanisms whereby the thyroid hormones are synthesized. Whether this interference is accomplished by blocking iodination of tyrosine or by inhibiting the oxidative coupling of iodotyrosines to form iodothyronines has not been determined (Astwood, 1955).

The administration of goitrogens to mammals results in thyroid hyperplasia and a distinct decrease in basal metabolic rate (Astwood *et al.,* 1943), but few investigations have been made on the effect of these agents in invertebrates. No studies have been reported on the effect of these chemicals on metabolic rate in planarians as evidenced by oxygen consumption.

The present work is a study of the effect of goitrogens on oxygen consumption in *Dugesia dorotocephala,* a common fresh water planarian.

Design of Experiment

Planarians were collected from Buckhorn Springs[2] in Murray County, Oklahoma, and kept in the laboratory for several weeks before this series of experiments was begun. The worms were maintained in aerated lake water in deep, white-enameled pans, at a temperature of 20° C.

Three goitrogens were used in this study, as follows: 0.005 *M* and 0.0025 *M* thiourea, 0.0005 *M* and 0.00025 *M* phenylthiourea, and 0.005 *M* and 0.0025 *M* thiouracil. These concentrations were found not to be toxic to planarians regenerating in the solutions for a period of one week.

Worms not fed for seven days were subjected to the higher concentrations of each chemical; lower concentrations were used for animals not fed for eight days before the beginning of the experiment. The choice of the seventh and eighth day after feeding was made on the basis of earlier experiments (Jenkins, 1960) which demonstrated that phase *C*, the period of most gradual decline of metabolic activity in planarians which occurs some time after feeding, was established in these animals after about the sixth day.

On the day that an experiment was begun, thirty of the largest specimens were chosen from one stock pan and examined with a binocular microscope, then divided randomly into six groups of five worms. Each group was thereafter treated as a unit. Six replicates were used for each series of experiments performed.

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[2] Acknowledgment and thanks are due to Oscar Lowrance, owner of Buckhorn Springs property, for permission to collect the planarians.

The first determination of oxygen consumption, on Day 0, was made with the six groups of animals in water. At the close of the readings the worms were weighed, then placed in labeled fingerbowls, each containing 100 ml. of the goitrogen solution to be used for that phase of the study. Readings on the second, fourth and sixth days were made with the animals in the same solution as that in which they had been living. On the sixth day, after readings had been made, three of the six groups of animals were returned to water, the other three being kept in the chemical. The final two measurements were made on the eighth and tenth days.

For determinations of oxygen consumption, the Warburg respirometer was used. After a thirty-minute equilibration period, readings were taken every half-hour for three hours. The respirometer was kept in a constant temperature room so that the experimental animals were subjected continuously to a temperature of 20° C. At the termination of the day's readings, each group of worms was weighed on a Roller-Smith torsion balance accurate to 0.2 mg. They were then returned to clean fingerbowls containing fresh solution.

Two series of oxygen consumption measurements on controls kept in water were made in a similar manner, one for worms starved seven days, and the other for those without food for eight days.

A second set of experiments was performed to test whether or not the goitrogens would cause an increase or decrease in the oxygen consumption of the planarians immediately after they were placed in the chemical. The same concentrations were used, and the procedure was similar to that outlined above, with the following exceptions: After the first measurement in water on Day 0, the worms were returned to water for 24 hours. For the second measurement, on Day 1, the worms were placed in reaction flasks prepared with the proper goitrogen, and measurements of oxygen consumption during the first 3½ hours in the chemical were made. The worms were then placed in the drug overnight, and the third and final readings taken after 24 hours' exposure.

RESULTS

Data secured with these treatments were calculated according to standard methods (Umbreit *et al.*, 1949) and the results expressed in microliters of oxygen consumed per gram of wet weight per hour. The values obtained with the six replicates were subjected to a statistical analysis of variance and found to be significant at $P < .001$.

Both thiourea and thiouracil depressed oxygen consumption slightly but not significantly below the usual gradual decline found in planarians subjected to prolonged starvation (Jenkins, 1960). A return to normal levels was established within four to six days in the animals exposed to the goitrogens for a prolonged period.

Planarians placed in 5×10^{-4} M phenylthiourea showed a significant fall in oxygen consumption within the first 3½ hours after they were placed in the goitrogen (Table I). In the animals kept in phenylthiourea for a prolonged period, a return to normal levels occurred within two to four days, followed by a gradual

TABLE I

Effect of goitrogens on oxygen consumption of planarians within 27½ hours after initial exposure

Treatments	μl. O_2/gm./hr.		
	Days		
	0	1	2
Water	140	147	142
Thiourea, 5 × 10^{-3} *M*	121	122	135
Thiourea, 2.5 × 10^{-3} *M*	120	117	127
Phenylthiourea, 5 × 10^{-4} *M*	143	116	127
Phenylthiourea, 2.5 × 10^{-4} *M*	138	105	119
Thiouracil, 5 × 10^{-3} *M*	141	143	136
Thiouracil, 2.5 × 10^{-3} *M*	132	140	133

rise which was significantly above normal by the sixth day, the maximum being reached on the eighth day. The effect was similar in the lower concentration, but less pronounced. A return to water slightly diminished the rise in oxygen uptake, but a return to normal levels was not established within four days.

The effect of the higher concentrations of goitrogens on the respiratory rate of the planarians as compared with the usual gradual decline exhibited by the water controls is illustrated in Figure 1.

DISCUSSION

The mechanism of goitrogenic action has not been completely elucidated, but current evidence favors the view that these compounds, by virtue of their reducing activity, produce their effect in the vertebrate thyroid by interfering with the conversion of iodide to iodine, presumably by inhibiting a peroxidase (Astwood, 1955), although the presence of the latter in the thyroid has not been demonstrated.

It is possible that the action of the goitrogens in depressing oxygen consumption in planarians is mediated through a similar mechanism. Although the fall in

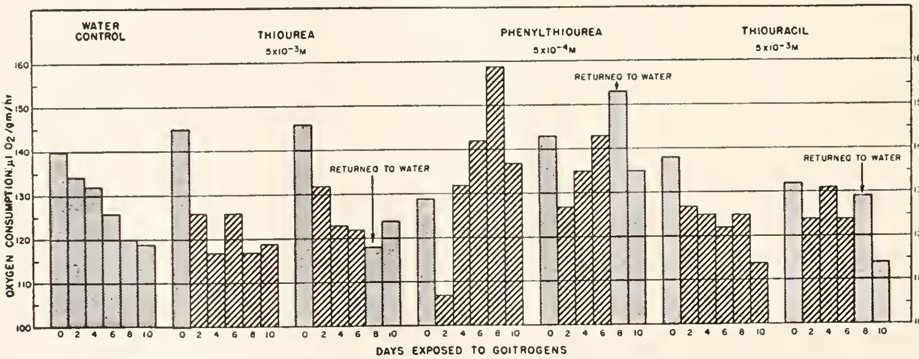

FIGURE 1. Effect of higher concentrations of goitrogens on oxygen consumption of planarians.

metabolic rate with thiourea and thiouracil was statistically insignificant, the depression in rate occasioned by the use of phenylthiourea was pronounced and continued for several hours. Jones and Wilson (1959) found the phenylthiourea-induced fall in the rate of oxygen consumption in *Cynthia* silkworm larvae and pupae was accompanied by an inhibition in the blood phenolase (tyrosinase) activity, and suggested the possibility of this enzyme being implicated in some way with respiration. Its action is blocked by a number of goitrogens, including thiourea, phenylthiourea and thiouracil.

Tyrosinase has not been demonstrated in planarians, but it is found in many invertebrates. Its presence in planarians is suggested, however, by reports of the effectiveness of thiourea compounds in causing eye depigmentation. Kambara (1954) found that pigment granules in eye-spots disappeared as a result of thiourea treatment, but skin pigment was not affected. His results were confirmed by Teshirogi and Maida (1956) and Kido and Kishida (1960). Jenkins (1959) noted that the formation of pigment was inhibited completely in the eyes of planarians allowed to regenerate in solutions of phenylthiourea at various concentrations. In the planarians returned to water, a steady development of pigment occurred. The colors appeared in the order commonly noted in melanin formation, namely tan, red to reddish-brown, and finally black. In view of these facts, it appears quite possible that the depressant action of phenylthiourea on oxygen uptake in planarians may be due to the inhibition of tyrosinase by the goitrogen.

The stimulatory effect of the phenylthiourea on planarian respiration, after prolonged exposure, is presumed to be due to the toxicity of the goitrogen. The reversal of the initial depression, which was followed by a pronounced increase in respiratory rate, was accompanied by some degree of cytolysis. Although an effort had been made to use only physiologically tolerated concentrations, in the 5×10^{-4} M phenylthiourea, on the tenth day after the worms had been put into the chemical, one worm had several small lesions and five others had one or two small lesions each. None of the worms in the lower concentration of the drug showed any evidence of similar injury; nevertheless, a similar but less pronounced rise in oxygen consumption shows that they were affected. The cause, under the circumstances, is presumed to be the same in both instances.

It might be of interest to add that a possible effect of goitrogens on sexual activity in planarians was noted. During the time the experimental animals were kept in the solutions, both copulation and cocoon deposition occurred. Six pairs of copulants were seen in the thiourea and five in thiouracil, but none was observed in either water or phenylthiourea. Ten cocoons were deposited by the thiourea-treated animals, and one by the water controls, but none was found in either of the other chemicals. Two of the ten cocoons were deposited in the Warburg reaction flasks while measurements were being made.

The ten cocoons were transferred to separate bowls of thiourea solution and kept for four weeks. By the fourth day they had begun to lose their original creamy whiteness, but complete darkening did not occur. No worms emerged from any one of the ten.

Summary and Conclusions

1. A study was made of the effect of the goitrogens thiourea, phenylthiourea and thiouracil on oxygen consumption in planarians.

2. Thiourea and thiouracil were found to have no statistically significant effect.

3. With phenylthiourea there was a marked initial depression, then a gradual return to normal levels followed by a significant rise. In the animals returned to water, oxygen consumption was not reduced to normal levels within four days.

4. It is suggested that the depressant action of the phenylthiourea may be ascribed to its ability to inhibit tyrosinase, while the stimulatory effect may well be due to the toxicity of the goitrogen.

5. A possible effect of thiourea upon sexual activity in planarians was noted.

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PHOTORECEPTOR CELL RESPONSE AND FLICKER FUSION FREQUENCY IN THE COMPOUND EYE OF THE FLY, LUCILIA SERICATA (MEIGEN)

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Compound eyes of insects function over a wide range of light intensity and speed of movement. At one extreme are diurnal species such as the honeybee, dragonfly and blowfly, which can orient visually to their surroundings while in rapid flight. At the other extreme are crickets and walking sticks, which are nocturnal and seldom or never fly. Autrum (1950) was the first to point out that the compound eyes of species at the two extremes differ in electrophysiological properties, specifically in properties of the electroretinogram (ERG). Autrum and co-workers (Autrum, 1950; Autrum and Stoecker, 1950; Autrum and Gallwitz, 1951) cited the following properties as characteristic of the honeybee, dragonfly and blowfly: (1) a diphasic ERG with positive on-effect and negative off-effect; (2) flicker fusion frequencies (FFF) ranging from about 180/sec. to over 300/sec.; (3) relatively low sensitivity to light; (4) high rate of dark adaptation. In contrast, Autrum (1950) cited these properties as characteristic of the cricket and walking stick: (1) a monophasic, cornea-negative ERG; (2) FFF lower than about 60/sec.; (3) relatively high sensitivity to light; (4) slow rate of dark adaptation. The two extremes were designated "fast" and "slow" eyes.

My own data are in good agreement with Autrum's on FFF, though not with respect to wave form of the ERG, sensitivity, and rate of dark adaptation (Ruck, 1958a, 1958b). Attention here is directed to the area of agreement, namely that FFF may be several times higher in certain diurnal insects than in certain nocturnal ones. The ultimate physiological problem in this area is to explain the molecular mechanisms underlying the differences in FFF, but first it is necessary to identify the anatomical structures in which the mechanisms operate. Autrum (1952, 1958) has proposed that two layers of cells—the photoreceptor cells and postsynaptic neurons of the *lamina ganglionaris*—are essential to the production of high FFF in the fast eye. According to this hypothesis, the electrical response of the receptor cells evokes an electrical response in the *lamina ganglionaris,* and the two electrical fields then interact in such a way that desensitizing changes associated with light adaptation are kept to a minimum. An eye in which such electrical interaction occurs is capable of responding to more flashes per unit time than an eye in which the interaction is absent. Autrum (1952, 1958) proposes that photoreceptor cell responses are fundamentally similar in fast and slow eyes, and that differences in FFF are produced by addition of the *lamina ganglionaris* response to the fast eye only.

If the proposed mechanism actually operates in the fast eye, removal of the *lamina ganglionaris* response should result in a decrease of the FFF to values characteristic of the slow eye. Autrum and co-workers assert that this is the case. However, the methods which they have used to remove the response of the *lamina ganglionaris* are subject to the criticism that that response could not, by the nature of the experimental operations, be proven to be the only one effected. Thus, surgical removal of the *lamina ganglionaris* (Autrum and Gallwitz, 1951) necessitates transection of the axons of the photoreceptor cells where they enter the *lamina ganglionaris*. The postoperative ERG's in such an experiment must indeed be dominated by responses of the photoreceptor cells, but the assumption that these responses are unaltered by surgery is questionable. Lowering the P_{O_2} (Autrum and Hoffmann, 1960) and application of nicotine (Autrum and Hoffmann, 1957) involved simultaneous exposure of photoreceptor cells and cells of the *lamina ganglionaris*. The assumption that only responses of the latter were affected is again questionable.

This paper is concerned with a method of isolating the photoreceptor cell response in the fast compound eye of the fly, *Lucilia sericata,* without altering appreciably the response of the *lamina ganglionaris*. It is found that the electrical response of the whole optic ganglion, of which the *lamina ganglionaris* is a part, actually produces no detectable potential difference across the layer of photoreceptor cells. The photoreceptor cell potential develops in the presence of the optic ganglion potential and is unaffected by it. The isolated photoreceptor cell potential has a FFF higher than 200/sec.

The electrical interaction hypothesis (Autrum, 1952, 1958) specifies that the electrical field associated with the response of the *lamina ganglionaris* acts to prevent or arrest the development of a sustained electrical response of the photoreceptor cells. In view of present findings that hypothesis appears to be untenable. The data suggest the alternative hypothesis that the photoreceptor cells of the fast eye are inherently capable of responding to more flashes per unit time than photoreceptor cells in the slow eye.

METHODS

Flies of the species, *Lucilia sericata,* were collected locally. Each fly was anaesthetized for about two minutes with CO_2 and fastened to a small platform with Tackiwax (Central Scientific Co.) as shown in Figure 1. A ring of wax was formed about the neck to immobilize the head. The platform was oriented so that the long axis of the body was vertical, the front of the head facing directly upward. The preparation was then positioned beneath a dissecting microscope. A small wax cup was built up about the cornea of the experimental eye. The wax was manipulated with the aid of a wire whose temperature was maintained electrically at the melting temperature of the wax. A group of anterior ommatidia was left exposed in the bottom of the cup, and within this area a piece of cornea about four facets wide and eight long was dissected free from the subcorneal tissues.

An indifferent electrode (E_3), consisting of an electrolytically tapered stainless steel wire, was inserted into the intact compound eye so that its tip lay just beneath the cornea. This electrode was supported by a mass of wax attached to the same platform which bore the fly. After this electrode was placed, the intact eye was covered with aluminum foil which shielded the eye from the stimulating light.

The fly and platform with E_3 in place were then mounted on a micromanipulator with the long axis of the fly still in the vertical position. The preparation was moved into position beneath the optical stimulator. The light source was a Sylvania glow modulator tube mounted in a flashlight barrel behind two lenses. The light beam converged to a spot focus and was directed vertically downward onto the preparation. A glass micropipette electrode (E_2), filled with 3 *M* KCl, was fixed to a second micromanipulator and aligned vertically. The tip diameter of E_2 was less than one micron. The tip of E_2 was positioned in the focus of the stimulating light. The fly was then racked upward on its manipulator until the tip of E_2 just entered the area from which the cornea had been removed. The fly was not moved again during the experiment.

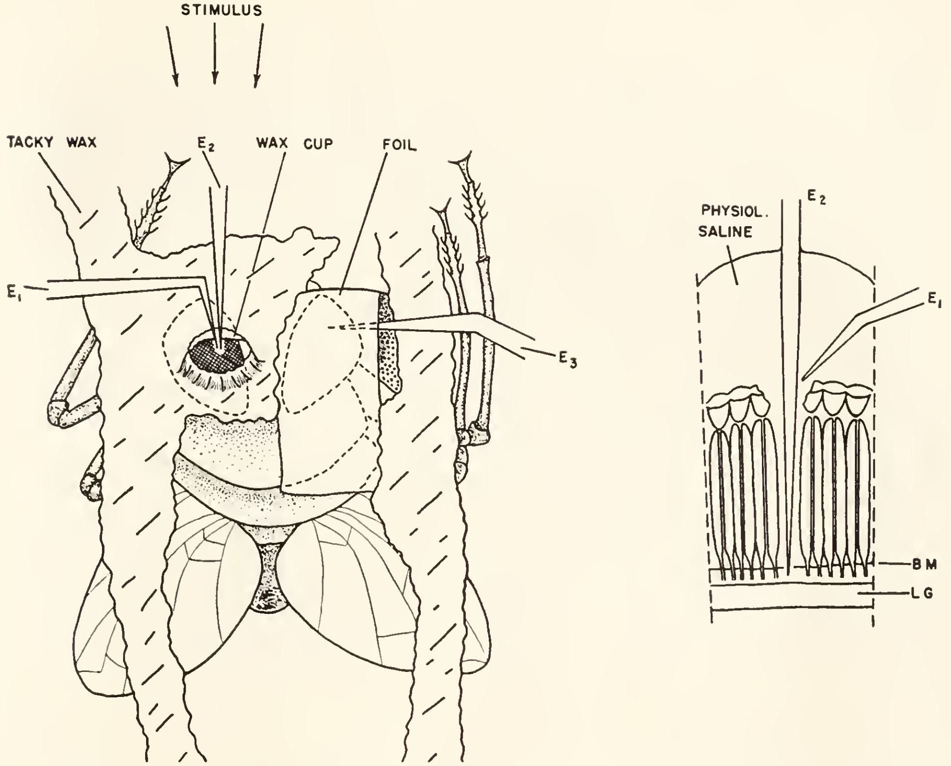

Figure 1. Left: *Lucilia sericata* in the experimental situation. Right: Diagrammatic section of the ommatidial layer to illustrate the positions of the electrodes in lead E_1–E_2. BM, basement membrane of the compound eye; LG, *lamina ganglionaris*.

A third electrode (E_1), consisting of a tapered stainless steel wire coated to the tip with an insulating varnish, was moved into position next to E_2 with the aid of a third micromanipulator. A small drop of physiological saline was added to the wax cup. This maintained good electrical contact between E_1, E_2 and the preparation, and also prevented drying. At the beginning of an experiment the tips of E_1 and E_2 were located at the level of the cornea. E_1 remained at this position. E_2 could be advanced into the ommatidial layer.

The electrodes were connected to the input of a Grass P6 DC amplifier. Leads

E_1–E_2 and E_1–E_3 could be selected alternately with the aid of a switch. Responses were displayed together with a stimulus signal marker on the screen of a dual beam oscilloscope.

The glow modulator light source drew current from a DC power supply which was controlled by a Grass S4 physiological stimulator. At the beginning of an experiment the stimulus frequency was set at a constant one flash per second with a stimulus duration of 1/8 sec. Lead E_1–E_3—cornea of experimental eye vs. an electrically indifferent site—was selected first. Stimulus intensity was adjusted to produce a diphasic ERG with a positive on-effect and a negative off-effect. Then lead E_1–E_2 was selected. Since E_1 and E_2 were both at the level of the cornea at the start of an experiment, no responses could be recorded until E_2 was advanced into the ommatidial layer. As E_2 advanced, an ERG appeared and increased in amplitude with depth. This ERG was quite different in wave form from the one recorded in lead E_1–E_3. It consisted primarily of a cornea-negative wave. When this wave attained its maximum amplitude, a scale reading on the micromanipulator bearing E_2 was taken. A previous scale reading corresponded to the level of the cornea.

The path actually traversed by E_2 was determined at the end of each experiment. The fly was left in place and most of the dorsal ommatidia were stripped away from the head. This exposed the ommatidia along the path which E_2 had followed. This layer of ommatidia was viewed laterally with the aid of a dissecting microscope and the experimental penetration of E_2 was re-enacted by moving it along the same path and to the same depth as in the experiment. The position of E_2 at its deepest penetration in the experiments cited below was always at approximately the level of the basement membrane (Fig. 1). This position is an easy one to locate when the electrode is advanced blindly into the eye, because it corresponds to the maximum amplitude of the cornea-negative potential in lead E_1–E_2. Just beyond this position the ERG undergoes drastic alteration of wave form. At some depth within the layer of the optic ganglion corresponding to the *lamina ganglionaris* (Fig. 1) ERG's in lead E_1–E_2 become essentially like those recorded in lead E_1–E_3. The *lamina ganglionaris* is visible in the freshly dissected preparation as a white band of nervous tissue located close to the basement membrane of the ommatidial layer.

ERG's were recorded first across the ommatidial layer (E_1–E_2) under stimulus conditions which produce the diphasic wave form in lead E_1–E_3, then across the whole head (E_1–E_3) without changing the stimulus conditions, and finally across the ommatidial layer again (E_1–E_2) to determine whether the preparation remained in the same physiological condition. Using this sequence, responses to single flashes and to light flickering at 100/sec. and 200/sec. were recorded.

Stimulus intensities are given as equivalent footcandles illumination produced at the cornea of the experimental eye. Approximate values of illumination produced by the glow modulator were calibrated by a method described elsewhere (Ruck, 1961a). All experiments were conducted at room temperature which varied between 23° and 25° C.

RESULTS

(1) Flies were mounted as shown in Figure 1. The stimulating light path was aligned with the visual axes of the anterior ommatidia. Preliminary responses

were observed using lead E_1–E_3. Stimulus duration was 1/8 sec., stimulus repetition rate one flash per second. The stimulus intensity was adjusted until the diphasic ERG illustrated in Figure 2,*b* was obtained. This is similar to the ERG Autrum (1950) describes as characteristic of the fast eye of the fly, *Calliphora*. Stimulus intensity was maintained constant at this level during the remainder of the experiment. Next, lead E_1–E_2 was selected. When both electrodes were at the level of the cornea, no response could be detected in this lead. E_2 was advanced until the ERG of Figure 2,*a* was recorded. The tip of E_2 then lay at about the level of the basement membrane of the ommatidial layer. The response across E_1–E_2 to a single 1/8-second flash (Fig. 2,*a*) consists of a sustained cornea-negative deflection plus a brief, cornea-positive on-deflection; this response is remarkably similar to responses obtained from the layer of photoreceptor cells in dorsal ocelli (Ruck, 1961a). A response to a light flickering at 100/sec. was recorded.

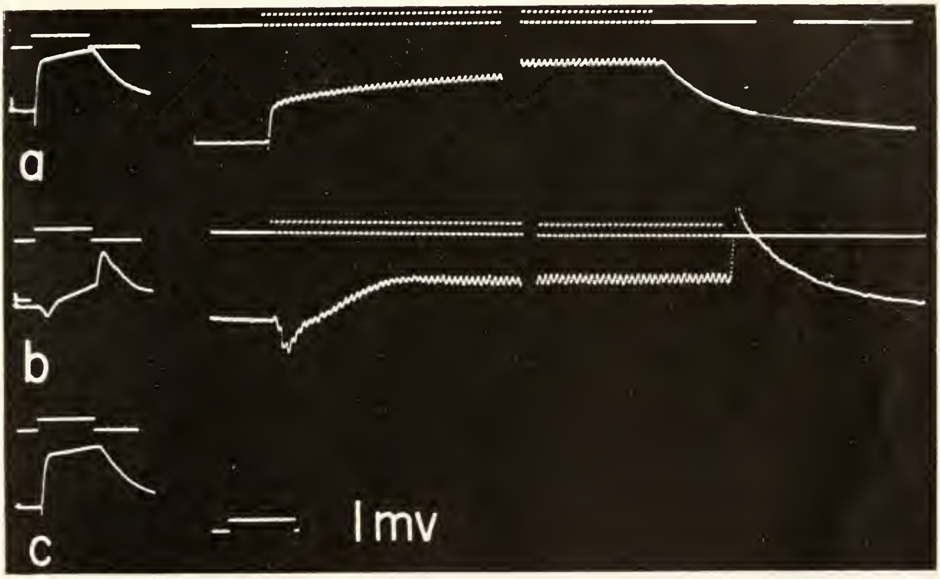

FIGURE 2. Comparison of ERG's in lead E_1–E_2 (records *a* and *c*) and lead E_1–E_3 (record *b*). For single flashes (left), stimulus duration is 1/8 second. Flicker frequency (right) is 100/sec. Stimulus intensity is constant (approximately 45 footcandles at the cornea) for all records. DC amplification.

Next, lead E_1–E_3 was selected. In response to the 1/8-second flash the ERG is markedly diphasic (Fig. 2,*b*). It consists of a cornea-positive on-effect, a slight upward cornea-negative swing during illumination, and a cornea-negative off-effect. The response to a 100/sec. flicker stimulus is also shown.

Finally a control response was recorded, using lead E_1–E_2 (Fig. 2,*c*) in order to demonstrate that the condition of the preparation had not changed during the experiment.

The ERG's of Figure 3 were obtained from another animal. Only responses to light flickering at 200/sec. are shown. In lead E_1–E_2 (Fig. 3,*a*) the ERG is a monophasic, cornea-negative potential upon which are superimposed small waves, one for each cycle of the stimulus. The recording obtained using lead E_1–E_3 (Fig. 3,*b*) also displays the responses to each cycle of the stimulus, but the wave form of the ERG is the diphasic type which Autrum (1950) described as characteristic of the fast eye. The recordings of Figures 3,*a* and 3,*b* were made at different amplifier gains so that the flicker potentials in Figure 3,*b* appear larger than those in 3,*a*. In order to compensate for this, a portion of each ERG was enlarged photographically (Figs. 3,*c* and 3,*d*) so as to make the vertical amplifications identical. At the same amplification the amplitudes of the flicker potentials are approximately equal. The experiment demonstrates that a flicker potential is as prominent in the absence of cornea-positive components of the ERG as in their presence.

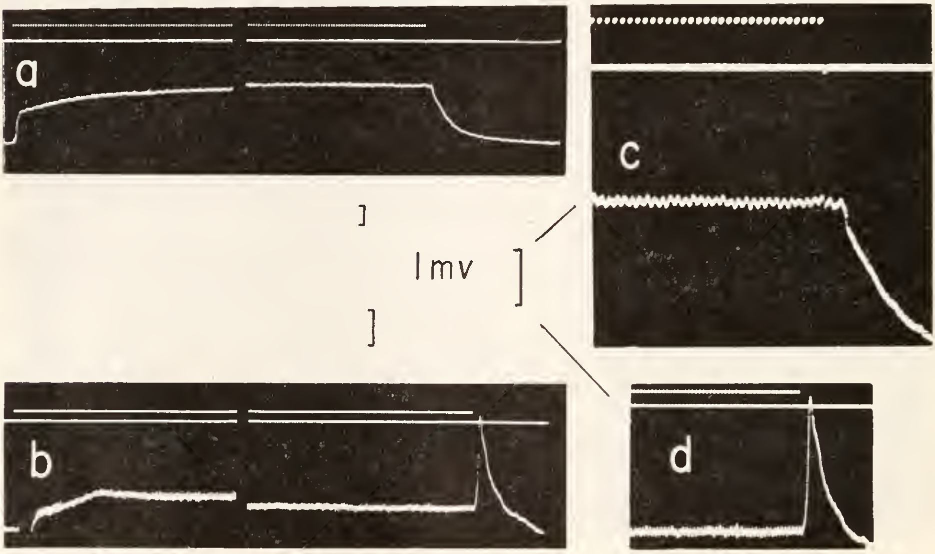

FIGURE 3. Responses in lead E_1–E_2 (records *a* and *c*) and in E_1–E_3 (records *b* and *d*) to light flickering at 200/sec. In *c* and *d* vertical amplifications of portions of responses in *a* and *b* have been made equal photographically. DC amplification.

Similar experiments were performed on 10 eyes. The only difference in results was that in five cases the brief, cornea-positive on-deflection in lead E_1–E_2 could be seen clearly (Fig. 2,*a*), and in the other five it could not (Fig. 3,*a*).

(2) All of the previous ERG's were recorded at the same stimulus intensity. The ERG's of Figure 4 illustrate the way in which wave form varies with stimulus intensity. All ERG's in Figure 4 were evoked by stimuli of 1/8-second duration, and all are steady-state responses to stimuli presented at a rate of 1/sec. Stimulus intensity increases from left to right in each row. In lead E_1–E_2 (Fig. 4, top row), the wave form is similar throughout the range of intensities. There is a sustained, cornea-negative potential plus a brief, cornea-positive on-deflection. The response simply increases in magnitude with stimulus intensity. Responses recorded with lead E_1–E_3 from the same eye immediately after the preceding series

are shown in Figure 4, middle row. The threshold response in E_1–E_3 is a monophasic, cornea-positive potential which does not appear at all in E_1–E_2. With increase in stimulus intensity it tends to overshoot the baseline as a negative off-effect. The brief, cornea-positive on-deflection seen in E_1–E_2 also appears in E_1–E_3. It is first visible in Figure 4,*b*, middle row. It has the same polarity in the two leads.

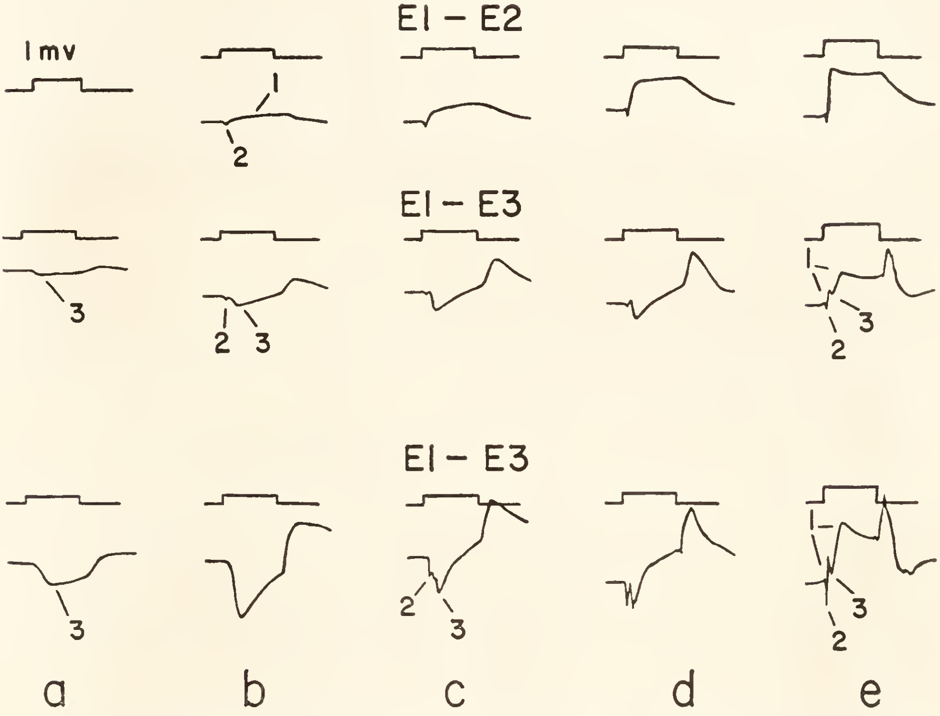

FIGURE 4. ERG's as a function of stimulus intensity. Leads indicated. Upper and middle rows from one eye, lower row from another. Stimulus duration, 1/8 second throughout. Stimulus intensity increases from *a* through *e*: *a*, 0.012; *b*, 0.12; *c*, 1.2; *d*, 46.1; *e*, 1200 foot-candles at the cornea. DC amplification.

Three components of the *Lucilia* ERG are numbered in Figure 4, using the same convention adopted in an analysis of the ocellar ERG of dragonflies and cockroaches (Ruck, 1961a). Components 1 and 2 appear in leads E_1–E_2 and E_1–E_3. Component 3 appears in E_1–E_3 only. The records of Figure 4, bottom row, illustrate a case in which components 2 and 3 were particularly conspicuous in lead E_1–E_3.

DISCUSSION

The cornea-negative component of the ERG (component 1, Fig. 4) appears in virtual isolation when only the ommatidial layer is included between the recording electrodes (E_1–E_2). One cornea-positive component (component 3, Fig. 4), which is prominent when optic ganglion and ommatidial layer both lie between the recording electrodes (E_1–E_3), is excluded in lead E_1–E_2. These results are consistent with the interpretation that component 1 originates in the photoreceptor cells, component 3 in some deeper lying structure of the optic ganglion. The fact that component 3 cannot be recorded in lead E_1–E_2 indicates that the process responsible for that component produces no detectable potential difference between the cornea and the basement membrane of the ommatidial layer. Therefore, the only electrical interactions between components 1 and 3 are simple algebraic summations of potential wholly dependent upon the locations of the recording electrodes and without physiological significance. On the assumption that components 1 and 3 correspond respectively to the receptor cell and optic ganglion potentials of Autrum, his hypothesis (Autrum, 1952, 1958) of the mechanism of the fast eye response appears to be untenable. That hypothesis specifies that the electrical field associated with the optic ganglion potential prevents the development of a sustained receptor cell potential.

The negative off-effect recorded in lead E_1–E_3 (Fig. 4, middle and bottom rows) does not appear in lead E_1–E_2 (Fig. 4, top row). This off-effect, which in Autrum's hypothesis (1952, 1958) is regarded as a sudden release from inhibition of the receptor potential, actually originates in the optic ganglion. The off-effect is regarded here to be part of component 3. The receptor potential, component 1, simply decays toward the baseline at "off."

Component 2 is thought to originate in the photoreceptor cells. It was recorded in five out of ten preparations, in each of which it appeared together with component 1 in lead E_1–E_2. Component 3 was never recorded in this lead. A very similar component, also designated component 2, was found in dorsal ocelli (Ruck, 1961a, 1961b, 1961c). Component 2 was interpreted tentatively as a depolarizing response of the axons of the photoreceptor cells. The present interpretation of component 2 of the compound eye of *Lucilia* is the same, and depends upon the ocellar data. That is, the interpretation is based upon comparative evidence and the concept of homology of structure and function of retinula cells in dorsal ocelli and compound eyes.

Extending the comparative argument, component 3 may be interpreted as a hyperpolarizing, postsynaptic potential which arises at the junctions between retinula cell axons and second order nerve fibers of the *lamina ganglionaris*. The negative off-effect, if homologous with the similar component of the ocellar ERG, is a depolarizing after-potential—a sign of postinhibitory excitation—of the second order neurons in which component 3 originates.

A physiological mechanism which satisfactorily explains the difference in FFF between fast and slow eyes will probably be concerned mainly with properties of the retinula cells. The time would appear to be ripe for comparative studies of the responses and ultrastructure of individual retinula cells. Naka (1961) has begun work of this type and has obtained intracellular recordings from retinula cells of *Lucilia* which are consistent with interpretations of components 1 and 2 presented here.

The author would like to thank his wife for preparing the figures in this paper. The work was supported by a grant to Dr. K. D. Roeder from the United States

Public Health Service. Some of the equipment used was obtained under a previous contract between the Army Chemical Corps and Tufts University.

SUMMARY

1. That part of the ERG which orginates in the retinula cells of the compound eye in *Lucilia sericata* was isolated without interfering with components originating in the optic ganglion. The retinula cell response is predominantly a sustained, cornea-negative wave, but in half the cases studied a brief, cornea-positive wave at "on" was also present.

2. Electrical events which originate in the optic ganglion produce negligible potential difference between the cornea and the basement membrane of the ommatidial layer. Therefore, an electrical effect, *per se*, of an optic ganglion process on the retinula cells is very improbable.

3. The electrical response of the retinula cells, uninfluenced by electrical fields produced by the optic ganglion, can resolve flicker stimuli of 200/sec. or higher.

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A STUDY OF THE EFFECTS OF URETHAN ON THE CLEAVAGE OF THE CHAETOPTERUS EGG. I. INHIBITION OF CLEAVAGE[1]

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Urethan (ethyl carbamate) is known to act as a potent antimitotic agent against cells derived from a wide variety of plant and animal tissues (Cornman, 1954). In addition, as a result of the pioneer work of Haddow and Sexton (1946), urethan has been used extensively in the chemotherapy of clinical and experimental tumors (Greenstein, 1954; Homburger and Fishman, 1953).

However, several lines of investigation have to date failed to produce conclusive information concerning the mechanism by which urethan inhibits cell division. Warburg (1956) suggested that urethan prevents cell division and arrests tumor growth of blocking cellular respiration. The theoretical basis of this hypothesis has generated a great deal of spirited debate in the literature (Weinhouse, 1955; Weinhouse *et al.,* 1956), but has not yet been proved experimentally. In addition there is some evidence that urethan may inhibit a biosynthetic pathway that is essential for cell division. In microorganisms and in tissue culture the administration of urethan or other carcinostatic substances results in the inhibition of cell division and the production of giant cells (Lasnitski, 1949; Sato, Belkin and Essner, 1956; Sirtori, Parvis and Pizzetti, 1955). Recent work has suggested that urethan may inhibit the biosynthesis of pyrimidines (Bresnick, 1960) and nucleic acids (Skipper, 1950; Skipper *et al.,* 1951). More experimental work is necessary, however, before the significance of these observations can be evaluated properly.

The present study was undertaken to investigate the possibility that urethan may be acting directly upon the physiological mechanisms responsible for the assembly of the mitotic apparatus. The eggs of marine invertebrates constitute an excellent experimental system for this type of work. Large numbers of cells can be readily obtained, which undergo division with almost perfect synchrony following fertilization. Experiments with very young embryos are presumably not complicated by problems of *de novo* net synthesis of proteins and nucleic acids (Brachet, 1957) or by problems of growth (Swann, 1957). However, these cells do convert storage proteins and nucleic acids into protoplasmic components, and this process may introduce complications.

Heilbrunn (1956) and his students have intensively studied the physical properties of the cytoplasm of marine eggs during the mitotic cycle. A sharp increase in relative cytoplasmic viscosity, the mitotic gelation, takes place just prior to the cytological appearance of the mitotic apparatus (Heilbrunn and Wilson, 1948). Mazia (1958a) has shown that the mitotic apparatus is formed by the mobilization of soluble phase macromolecules that are already present in the unfertilized egg. Eggs treated with a wide variety of carcinostatic substances do not divide, and the mitotic gelation preceding the formation of the mitotic apparatus does not take place (Heilbrunn and Wilson, 1957; Heilbrunn *et al.*, 1957). The mitotic gelation may, therefore, reflect a generalized aggregation of macromolecules out of which the ordered structure of the mitotic apparatus is ultimately formed.

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The experiments to be described indicate that in marine eggs urethan acts directly upon the mechanical structures of the dividing egg, the mitotic apparatus and the cortex. These experiments have been reported briefly elsewhere (Schuel, 1957; Schuel, 1958; Schuel, 1959).

I am indebted to the late Dr. L. V. Heilbrunn for suggesting this problem to me, and for his continual help and encouragement during the course of this investigation. I also want to thank Dr. Lester Goldstein for his guidance in the preparation of the manuscript.

Dedicated to Dr. L. V. Heilbrunn, my teacher and my friend.

MATERIALS AND METHODS

The eggs of the marine annelid *Chaetopterus pergamentaceus,* which are available at Woods Hole, Mass., during the summer months, were used.

Gametes can be readily obtained from the worms by cutting the parapodia (for details, see Costello *et al.*, 1957). The eggs were filtered through several layers of cheesecloth and washed at least three times with sea water before being used. The eggs were maintained at 21° C. in a constant-temperature bath for the duration of the experiments. At this temperature about 50% of the eggs have divided at 56 minutes following insemination. In order to determine the percentage of divided cells, samples of 50 eggs were counted. This procedure made it possible to make rapid measurements of events taking place in populations of eggs maintained under a variety of experimental conditions, and the results from one experiment to another always indicated a definite and consistent trend.

Estimates of relative cytoplasmic viscosity were made according to the method described by Heilbrunn and Wilson (1948). A force of approximately 2250 times gravity was applied to the eggs by means of the hand centrifuge, and "relative viscosity" was judged by the number of seconds of exposure to this force required to produce a definite shift of light and heavy granules to opposite poles of the cell. During much of the time between insemination and first cleavage, 7 seconds are required to produce the visual impression of zones in the *Chaetopterus* egg with a force of this magnitude. About 27 minutes following insemination the cytoplasmic viscosity begins to increase, so that at 30 minutes about 14 seconds are needed for zone formation. The relative cytoplasmic viscosity remains high until the mitotic apparatus appears about 10 minutes later. Viscosity measurements must be made during this brief period in order to observe if urethan affects the mitotic gelation. After the appearance of the mitotic apparatus, the viscosity returns to the basal level and rises sharply once again just prior to and during cytokinesis.

In order to visualize the mitotic apparatus within these eggs, cytological preparations were made with the help of Miss Jeanette Rose and Miss Jane Lehv. The eggs were incubated to the desired stage of the cleavage cycle, fixed in Bouin's fluid, dehydrated, sectioned (5 to 7 μ thick), stained with Heidenhain's or Harris' hematoxylin and mounted on permanent slides.

RESULTS

I. Inhibition of division and the mitotic gelation

Chaetopterus eggs were put into solutions of urethan (ethyl carbamate) 5 minutes after insemination, and the results of this treatment on cell division and relative cytoplasmic viscosity were observed (Table I). Preliminary experiments showed that urethan had to be present in concentrations greater than 0.5% in order to inhibit cell division. In supra-threshold concentrations of urethan the increase in relative cytoplasmic viscosity at 30 minutes, the mitotic gelation, was completely inhibited. The cytoplasmic viscosity of these eggs failed to rise above the basal level of 8 units and perhaps was even reduced. In 0.25% urethan, a subthreshold concentration, the mitotic gelation took place normally and cleavage was not affected. There thus appears to be a correlation between the prevention of the mitotic gelation and inhibition of cytokinesis in this system.

TABLE I

The effect of urethan on cleavage and the mitotic gelation

Experiment	% Concentrated urethan	Relative viscosity at 30 minutes	% cleavage at 60 minutes
1	0.00	more than 14	98
	1.00	less than 10	0
	2.00	less than 10	0
	3.00	less than 10	0
2	0.00	more than 14	100
	1.00	less than 10	0
	2.00	less than 10	0
	3.00	less than 10	0
3	0.00	more than 14	92
	0.25	more than 14	94
	1.00	less than 8	0

The exposure to urethan was begun at 5 minutes after insemination and continued for the duration of the experiment.

Relative viscosity indicated by the number of seconds of exposure to a force of 2250 times gravity required to produce a shift of light and heavy granules to opposite poles of the cell.

II. Recovery of eggs on return to sea water

The urethan inhibition was reversible, at least in some cases. Eggs were put into solutions of urethan 5 minutes after insemination. At the end of an hour, when all the control eggs had already divided, the inhibited eggs were returned to normal sea water and washed at least three times. The percentage of cleaved eggs was determined 15 minutes later. During this period relative cytoplasmic viscosity was estimated by means of the hand centrifuge. The results of typical experiments can be seen in Table II. At the end of 15 minutes almost all those eggs that had been in 1% urethan had divided, while only a portion of the eggs removed from 2% urethan recovered. Apparently the system is poised and ready to form the mitotic apparatus and complete division in the presence of urethan, and does so as soon as the inhibitor is washed away (see section IV below). Under these experimental conditions the inhibition by 3% urethan could not be reversed in any of the eggs. Upon return to sea water the 1% and 2% urethan-treated eggs divided into two blastomeres with the characteristic polar lobe, but the cells appeared to be somewhat "ragged" in appearance. The recovered eggs continued to divide and developed into very abnormal swimming larvae.

TABLE II

Recovery of inhibited eggs on return to normal sea water

Experiment	% urethan	Relative viscosity after 10 minutes	% cleavage after 15 minutes
1	1.0	more than 18	94
	2.0	more than 14	16
	3.0	less than 10	0
2	1.0	more than 16	88
	2.0	more than 20	16
	3.0	less than 10	0

Eggs were exposed to urethan 5 minutes after insemination and returned to normal sea water 55 minutes later.

Relative viscosity indicated by the number of seconds of exposure to a force of 2250 times gravity required to produce a shift of light and heavy granules to opposite poles of the cell.

Relative cytoplasmic viscosity determinations revealed somewhat surprising results. During the recovery period one might have expected the eggs to undergo cyclic viscosity alterations similar to those taking place normally during the mitotic cycle, a pre-mitotic gelation followed by a pre-cytokinetic solation and another sharp gelation just prior to and during cytokinesis. Several minutes following return to sea water the viscosity began to increase rapidly and sharply, culminating in cytokinesis within approximately 15 minutes. The maximum levels reached were considerably higher than those normally encountered during the mitotic gelation, but an accurate evaluation of the limits of this increase and its time course was not made because the cells were changing too rapidly. The pre-cytokinetic solation was thus absent, and the sharp increase in viscosity resembles closely the changes that have been reported by Heilbrunn and Wilson (1948) in the *Chaetopterus* egg during cytokinesis.

When the eggs were kept in urethan for longer periods of time, the first division following return to normal sea water was sometimes multipolar. In order to study this phenomenon quantitatively, eggs were put into urethan at 5 minutes after insemination and were returned to sea water at 30, 60, 90 and 120 minutes after fertilization. The eggs returned to sea water at 30 minutes showed a cleavage delay of perhaps 5 minutes, and divided into two blastomeres. The eggs that were exposed to urethan for longer periods of time divided in about 15 to 20 minutes following return to sea water. At 60 minutes after insemination the controls were in the two-cell stage, and the eggs removed from urethan at this time divided into two blastomeres. By 90 minutes the controls had reached the four-cell stage, and the eggs removed from urethan at this time primarily divided directly into four blastomeres. Finally, the controls were in the eight-cell stage by 120 minutes, and the eggs removed from urethan at this time primarily divided into eight blastomeres. It thus appears that the eggs removed from urethan will divide directly into the number of cells that would have been produced by that time had there been no inhibitor present.

TABLE III

Exposure of eggs to urethan at various times during the cleavage cycle

Experiment	1% urethan added at minutes after insemination	% eggs going through	
		First cleavage	Second cleavage
1	No addition	97	91
	10	0	0
	20	0	0
	30	0	0
	40	0	0
	50	5	0
	60	98	6
2	No addition	94	88
	10	0	0
	20	0	0
	30	0	0
	40	0	0
	50	6	0
	60	88	2

III. Exposure of eggs to urethan at various stages of the cleavage cycle

The previous experiments have demonstrated that urethan inhibits the mitotic gelation and prevents cleavage. Was the mitotic gelation then the critical step in the division cycle for urethan inhibition? In order to study this question eggs were put into 1% urethan at 10-minute intervals during the first hour following insemination. It is clear (Table III) that urethan prevented eggs from dividing even after they had completed the mitotic gelation. All the eggs in the controls divided within 58 minutes after insemination. Second cleavage was prevented in those eggs that had completed first cleavage when they were exposed to 1% urethan (beginning at 60 minutes after fertilization). The small number of second cleavages observed in these experiments probably represented eggs that had divided directly into four blastomeres during the first cleavage cycle. A small percentage of multipolar cleavages may sometimes be observed in the controls.

It appears that there is no critical point in the mitotic cycle of these eggs where urethan can no longer block that division but inhibits the next. The existence of such a critical point has been reported by Mazia (1958b) with mercaptoethanol, and by other workers with diverse chemical and physical agents (for references see Swann, 1957).

However, it remained to be seen whether division could be completed by eggs which had begun to form cleavage furrows at the time they were exposed to urethan. In order to answer this question, fertilized eggs were incubated in sea water until about half of the population exhibited cleavage furrows, and were then exposed to a 1% solution of urethan. Table IV summarizes the results of these experiments. At first the furrowing process appeared to continue, since two minutes later all the eggs seemed to have formed two quite distinct blastomeres and the characteristic polar lobe. However, the furrows soon began to regress and the cells reassumed a spherical shape, so that 15 minutes later only a small proportion of the eggs had completed cleavage planes. These few eggs probably had already completed cytokinesis at the time they were treated with urethan.

TABLE IV

Reversal of cleavage furrows by urethan

% urethan	% cleavage furrows 60 min. after fertilization	% cleavage furrows 15 min. later
0.0	100	100
1.0	100	40
0.0	90	82
1.0	86	9
0.0	98	100
1.0	96	14
0.0	98	98
1.0	92	40

Eggs were exposed to 1% urethan when about half the population exhibited cleavage furrows (approximately 56 minutes after insemination).

IV. Effect on the morphology of the mitotic apparatus

These results raise the problem of the fate of the organized structure of the mitotic apparatus in the presence of urethan. Normally the mitotic apparatus (centrioles and astral rays) is first visible in the *Chaetopterus* egg about 40 minutes after fertilization at 21° C. (Heilbrunn and Wilson, 1948). The experiments described above (section I) showed that eggs put into urethan at 5 minutes after insemination did not undergo the mitotic gelation and did not divide. One would predict that the mitotic apparatus was not formed.

This expectation was confirmed by the following experiment. Eggs were put into 1% urethan 5 minutes after insemination and fixed 40 minutes later. Control eggs fixed at the same time (45 minutes after insemination) appeared to be primarily in late prophase. The centrioles and the astral rays were sharply delineated at the poles of the cell while the nuclear membrane was in the process of breaking down. A few of the cells were in early metaphase. In the urethan-treated eggs no trace of the mitotic apparatus could be seen. In addition, a considerable number of these eggs still contained male and female pronuclei. Urethan at this concentration apparently retards but does not prevent the fusion of the pronuclei. Similar results have been obtained with other narcotics (Painter, 1918; Wilson, 1901).

It has also been shown (section III) that fertilized eggs did not divide when put into urethan after the mitotic gelation had taken place or after the mitotic apparatus had formed. In this case would the organized structure of the mitotic apparatus be destroyed or would it prove to be morphologically intact but functionally inactive? To resolve this question, eggs were put into 1% urethan 45 minutes after insemination, and were fixed 5 minutes later. Control eggs fixed at the same time were in metaphase and early anaphase. In the urethan-treated eggs none of the structural components of the mitotic apparatus could be seen. A small oval-shaped area, apparently free of granules or other structures, was observed near the center of the eggs, and this probably represented the location of the destroyed spindle. In addition, no intact nuclei were observed, suggesting that urethan failed to inhibit the physiological mechanisms responsible for the dissolution of the nuclear membrane.

Fertilized eggs that had been treated with 1% urethan divided in about 15 minutes after return to sea water (section II, above). During the recovery period there was a sharp and rapid increase in relative cytoplasmic viscosity (Table II), similar to the pattern observed by Heilbrunn and Wilson (1948) in the telophase egg. These results suggested that cytokinesis might be taking place without the mitotic apparatus. To resolve this point, eggs were exposed to 1% urethan 5 minutes after insemination and returned to sea water 55 minutes later. Aliquots of these eggs and eggs remaining in 1% urethan were fixed 10 minutes later. The eggs that had been returned to sea water contained a normal mitotic apparatus. This is an additional confirmation of numerous observations that marine eggs reconstitute the mitotic apparatus and divide after being removed from a variety of inhibitors (Boveri, 1897; Chambers, 1938; Harvey, 1927; Hertwig, 1890; Mazia, 1959; Pease, 1941; Runnström, 1930; Wilson, 1901). The eggs left in 1% urethan contained nuclei and showed no sign of the mitotic apparatus. The nuclear cycle apparently is not inhibited by urethan.

V. Effect on the cortex

It has been proposed that the cortex may play an essential part in the mechanics of cytokinesis (Marsland, 1956; Swann and Mitchison, 1958). In addition, many of the chemical and physical agents that alter the normal physiological activities of the cell also alter the physical characteristics of the cortex (Heilbrunn, 1956). A study was therefore undertaken of the effect of urethan on the rigidity of the cortex. The method of Wilson (1951) was employed. Eggs were put into urethan 5 minutes after insemination and the cortical rigidity was determined 5 minutes later. A centrifugal force of approximately 9300 times gravity was applied to the eggs for 60 seconds by means of the hand centrifuge. An egg was considered to have a "clear" cortex when the continuous string of cortical granules was disrupted. The results of these experiments appear in Table V. In 1% urethan the eggs were almost completely devoid of cortical granules. In a small number of control eggs the continuous string of cortical granules was broken, but even in these cases a large number of granules usually remained in the cortex.

In 0.125% urethan the cortical rigidity was equal to that of the controls. At this concentration cell division and development take place normally.

The inability of the eggs to complete cytokinesis upon exposure to urethan then may result from the destruction of the mitotic apparatus and liquifaction of the cortex.

VI. Proteolytic activity during the mitotic gelation

Heilbrunn (1956) suggested that cellular sol-gel transformations, such as the mitotic gelation, are accomplished by a two-step process similar to blood coagulation: an activation stage, dependent on calcium ions, and a calcium-independent polymerization stage, the latter presumed to be mediated by proteolytic enzymes. Gross (1954) demonstrated that the addition of ionic calcium to decalcified sea urchin egg homogenates activated a proteolytic enzyme and initiated an aggregation of nucleoproteins. Lundblad (1954) has shown that increased proteolytic activity is present in sea urchin eggs for the first 10 to 15 minutes after fertilization. This is the period when the mitotic gelation takes place in sea urchin eggs (Wilson, 1950). An attempt was therefore made to demonstrate protease activity during the mitotic gelation in *Chaetopterus* eggs, and then to determine whether urethan would affect the activation or activity of this system.

TABLE V

The effect of urethan on cortical rigidity. Proportion of eggs exhibiting a "clear" cortex

Experiment	Control	1.0% urethan	Experiment	Control	0.125% urethan
1	3/10	9/10	1	1/10	1/10
2	1/10	8/10	2	0/10	3/10
3	3/10	8/10	3	1/10	1/10
4	0/10	7/10	4	2/10	3/10
5	3/10	7/10	5	3/10	3/10
Average	20%	78%	Average	14%	22%

Analysis of the data by means of the Chi Square test for the difference between two proportions indicates that:

1. Comparing the two control series, *P* is less than 0.5, therefore the difference is probably not significant.
2. Comparing the control with 1% urethan, *P* is less than 0.01, therefore the difference is probably very significant.
3. Comparing the control with 0.125% urethan, *P* is less than 0.5, therefore the difference is probably not significant.
4. Comparing 1% with 0.125% urethan, *P* is less than 0.01, therefore the difference is probably significant.

Eggs were exposed to urethan 5 minutes after insemination, and 5 minutes later they were exposed to a centrifugal force of approximately 9300 times gravity for 60 seconds. An egg was considered to have a "clear" cortex when the continuous string of cortical granules at the edge of the cell was disrupted.

Because a considerable amount of material is necessary for an enzyme assay, the eggs of four or five ripe females were used. One aliquot of eggs was put into 1% urethan 5 minutes after insemination, and it was maintained, together with a control, until the desired stage of the mitotic cycle was reached (25 to 32 minutes after insemination). At this point the eggs were rapidly frozen in a bath of dry ice and ethylene-glycol and were then dried under vacuum. The powder was extracted in distilled water or 0.1 *M* phosphate buffer at about 5° C. for periods of one to three hours. The extracts were centrifuged and the supernatants assayed for proteolytic activity according to the standard method of Anson (1938), which involves the colorimetric determination of acid-soluble tyrosine. Incubation periods ranged from one to five hours at 25° C.

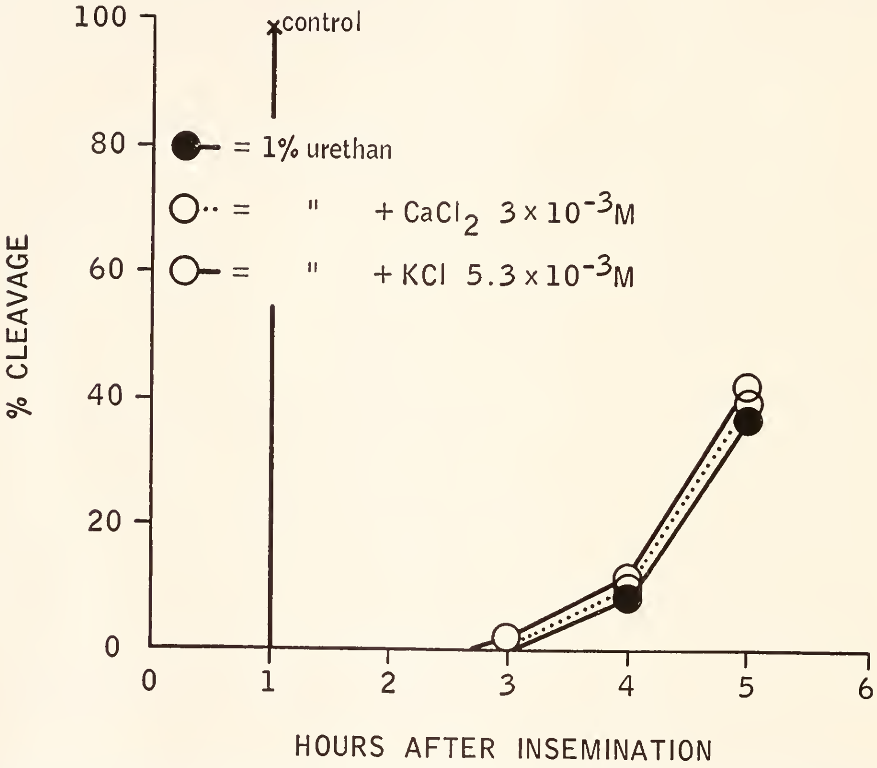

Figure 1. Attempt to overcome urethan inhibition of division by means of excess calcium and potassium. Eggs exposed to urethan-containing solutions 5 minutes after insemination.

The results of these experiments were completely negative. No proteolytic activity could be detected over a pH range of 3.6 to 8.0 (using acetate and phosphate buffers at 0.1 *M*) with either casein or bovine hemoglobin as substrates. In addition, tryptic digestion of casein, using Difco partially purified trypsin, was not inhibited by urethan over a concentration range of 1% to 7%.

VII. Attempts to overcome the urethan-induced inhibition of division

There is at present considerable evidence implicating multivalent cations as the "triggers" of cellular sol-gel transformations which are similar to those involved in mitosis and cytokinesis (Anderson, 1956; Heilbrunn, 1956; Gross, 1957). It was thought that the application of multivalent cations might overcome the urethan inhibition, thus establishing the stage at which the gelation process is blocked by urethan.

The organic cations were dissolved directly in a 1% solution of urethan in sea water. Isotonic solutions of the inorganic cations containing 1% urethan were mixed with 1% urethan solutions in sea water to produce the desired concentrations.

Early experiments (Schuel, 1958) indicated that small quantities of cations (inorganic calcium, histamine, protamine, arginine and lysine) could overcome the urethan inhibition. When fertilized eggs were kept in 1% urethan, very few of the eggs began to divide until about three or four hours after division had taken place in the controls. The addition of these cations made it possible for more of the eggs to divide while still in urethan. Thus, at two hours after insemination, from 10% to 30% of such eggs had already divided.

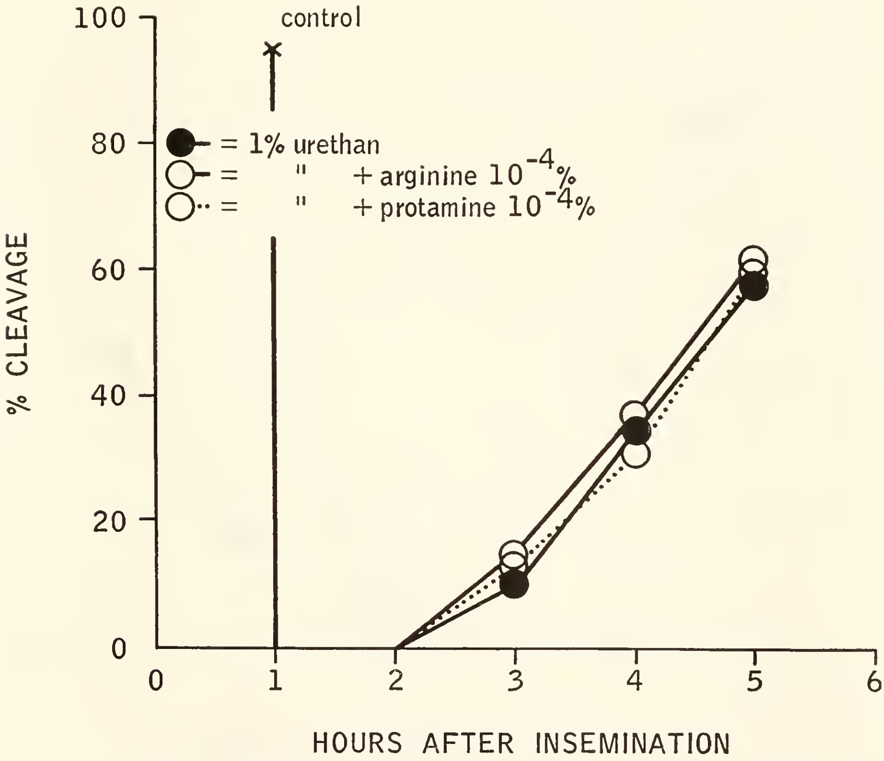

FIGURE 2. Attempt to overcome urethan inhibition of division by means of arginine and protamine. Eggs were exposed to urethan-containing solutions 5 minutes after insemination.

Not all of these promising results could be reproduced. In all subsequent experiments calcium, arginine and protamine failed to accelerate the recovery of eggs in urethan (Figs. 1 and 2). It was hoped that potassium might relieve the inhibition by replacing the calcium bound in the cortex, and thus liberate it for action in the interior of the cell (Heilbrunn, 1956). This too proved to be unsuccessful (Fig. 1). In the presence of higher concentrations of potassium ions, the eggs did not divide at all. Histamine and histidine, on the other hand, did accelerate recovery. The results of a typical experiment can be seen in Figure 3.

The possible role of proteolytic enzymes in cellular sol-gel transformations has already been discussed. The addition of small quantities of chymotrypsin ("Worthington 3X," crystals) or of trypsin ("Worthington lyophilized," crystals) accelerated the recovery of the eggs inhibited by 1% urethan (Fig. 4). Since calcium is believed to be bound to lipoprotein in the cortex of the cell (Heilbrunn, 1956), it was thought that lipase might also tend to relieve the urethan inhibition. Wheat germ lipase (Worthington) proved to be effective (Fig. 4). In one experiment the eggs were incubated in a mixture of trypsin and lipase (0.01 mgm./ml. each). The results were identical with those obtained by trypsin alone. However, after 24 hours the eggs were almost completely digested in the trypsin-lipase mixture, while they remained intact when incubated with either of these enzymes

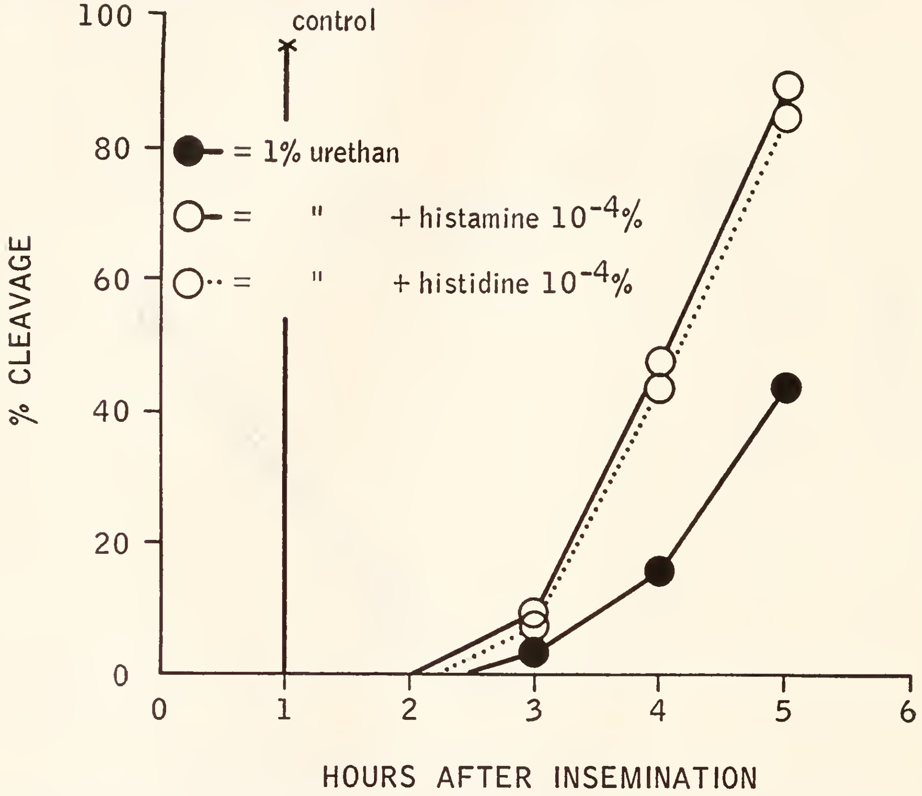

FIGURE 3. Attempt to overcome urethan inhibition of division by means of histamine and histidine. Eggs were exposed to urethan-containing solutions 5 minutes after insemination.

alone. Ribonuclease ("Worthington 3X," crystals) had no effect on the urethan-treated eggs.

In no case, however, was there more than a temporary recovery. The eggs divided a few times to produce grape-like clusters of cells but did not develop into larvae.

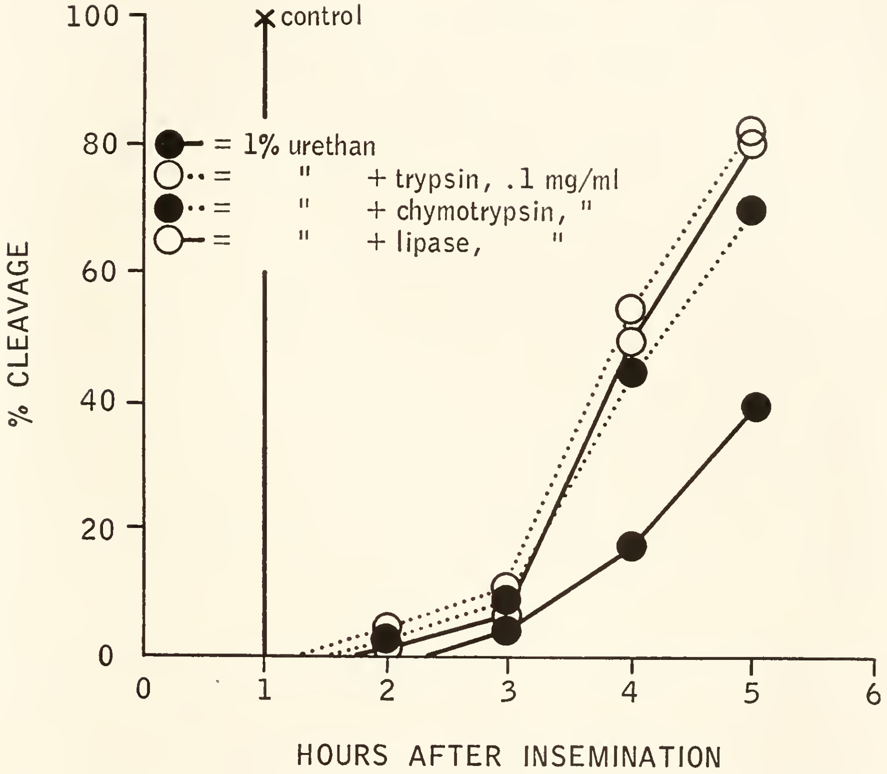

Figure 4. Attempt to overcome urethan inhibition of division by trypsin, chymotrypsin, and lipase. Eggs were exposed to urethan-containing solutions 5 minutes after insemination.

Discussion

Several workers have attempted to explain the action of urethan as a mitotic poison in terms of an inhibition of energy metabolism (McElroy, 1947; Fisher and Henry, 1944; Warburg, 1956). Bullough (1952) suggested that the cell accumulates an energy reservoir during interphase by means of respiration and glycolysis, sufficient to maintain itself during the entire division process. Until this storage is completed the division does not begin, but once begun a division is completed at normal speed in almost any circumstance short of the death of the cell itself. Bullough's hypothesis is based, in part, on observations that the application of many physical and chemical inhibitors to cells during mitosis or cytokinesis is without effect on that division but blocks subsequent divisions.

Swann (1957) discussed this concept at considerable length. However, Gelfant (1960) has recently questioned the validity of Bullough's experiments.

Mitotic arrest can be produced at any stage of the division cycle in the *Chaetopterus* egg by the application of 1% urethan. It is unlikely that these observations could be explained in terms of a depression of respiration or an inhibition of the filling of a hypothetical energy reservoir. While it is true that sea urchin eggs are unable to maintain the organized structure of the mitotic apparatus or complete cytokinesis in the total absence of oxygen (Harvey, 1927), these eggs are able to divide under very low oxygen tensions. Inhibition of mitosis is observed only after respiration is reduced to 20% to 30% of the normal rate by very low oxygen tensions or carbon monoxide (Amberson, 1928; Krahl, 1950). Apparently when respiration is depressed to such an extent, the eggs are unable to generate enough ATP to complete the division process (Barnett, 1953). However, urethan prevents the division of these eggs at concentrations that have little effect on respiration (for references see Krahl, 1950).

The production of multipolar divisions following removal of the urethan suggests that the metabolic events preceding mitosis, chromosome duplication for example, are not the primary site of the inhibition. Apparently the eggs will divide directly into the number of cells that would have been produced by that time had there been no inhibition. Similar observations have been made by other workers (Chambers, 1938; Gross and Spindel, 1960a; Marsland, 1938; Mazia and Zimmerman, 1958; Wilson, 1901). Recently Mazia *et al.* (1960) have reported that mercaptoethanol prevents the duplication of centrioles but does not inhibit the splitting and separation of centers already duplicated, thus enabling potential poles to be realized as actual poles in multipolar divisions. A similar kind of study should be made in the future of the effects of urethan on centriole duplication and migration.

The experimental evidence indicates that urethan may be acting directly upon the mechanical structures of the dividing cell and upon the assembly of these structures. The relative cytoplasmic viscosity determinations show that urethan does not allow the mitotic gelation to take place, and the eggs cannot divide. Furthermore, the mitotic apparatus cannot be formed. It is still not clear, however, just which stage of this biochemical process is vulnerable to attack by urethan.

An attempt was made to resolve this question by means of the experiments in which cations and enzymes were used in an attempt to relieve the urethan inhibition of division. It had been suggested that either calcium, arginine or protamine might function as initiators of macromolecular aggregation reactions leading to the formation of the mitotic apparatus (Anderson, 1956; Gross, 1957; Heilbrunn, 1956). However, these materials do not relieve the block to division produced by urethan, while histidine and histamine appear to be effective in hastening division in the presence of urethan.

Heilbrunn (1956) proposed that cellular sol-gel transformations, such as the one leading to the assembly of the mitotic apparatus, might be essentially a two-step process resembling the coagulation of blood. The protease assays of the eggs were conducted in order to test this hypothesis, as well as to investigate a possible point of attack by urethan. In this case negative results cannot be considered to be conclusive. *Chaetopterus* eggs may not be the best material for these experiments since the enzyme may be active for only a very short time. Lundblad

(1954) has demonstrated the transient activity of several proteases during the first cleavage cycle in sea urchin eggs, and they might prove to be a more satisfactory material with which to study this aspect of the problem.

The application of trypsin or chymotrypsin appears to enable the eggs to divide more rapidly in the presence of urethan. Goldstein (1953) demonstrated that trypsin is able to penetrate the *Chaetopterus* egg. These enzymes may act by mediating the second stage of the aggregation reaction within the eggs, and thus enable mitosis to continue. Alternatively, they might digest some intracellular proteins, thus releasing large amounts of organic or inorganic cations. Lipase might act by attacking the lipo-protein complex which is thought to bind calcium in the cortex (Heilbrunn, 1956).

In this connection the experiments concerning the reversal of the urethan inhibition on return to sea water are very suggestive. The eggs readily re-form the mitotic apparatus prior to division. Moreover the changes in cytoplasmic viscosity during the recovery period are similar to those observed in cytokinesis. Apparently the system is primed and ready to complete division in the presence of urethan, and does so as soon as the inhibitor is removed. Urethan thus may be affecting some chemical bonds that are essential for the polymerization of the spindle proteins.

If this is true, one might also expect urethan to destroy the mitotic apparatus after it has been formed. The cytological observations reported above show that this is indeed the situation. The mitotic apparatus is a very labile structure, and is readily dispersed by mechanical agitation (Chambers, 1938; Wilson, 1901), hydrostatic pressure (Pease, 1941), temperature shocks (Boveri, 1897) and narcotics (Swann, 1954; Wilson, 1901). These observations suggest that some kind of relatively weak chemical bond may play an important role in the maintenance of the organized structure of the mitotic apparatus.

A possible clue to the chemical mechanisms involved may be found in the recent experiments with heavy water and marine eggs (Gross and Spindel, 1960a, 1960b). Deuterium oxide inhibited division at any stage of the mitotic cycle prior to the completion of cytokinesis. The eggs in heavy water were all arrested in the stage of entry, and a gelation of the cytoplasm was also observed. These are the results one would expect with a primarily hydrogen-bonded structure. The urethan effects that have been found in this study (prevention of the mitotic gelation, prevention of the assembly of the mitotic apparatus, dispersion of the mitotic apparatus after it had been formed) may be reflections of an interaction with the hydrogen bonds that bind together the macromolecular components of the mitotic apparatus. In this connection, observations indicating that urethan may rupture hydrogen bonds in proteins are very suggestive (Johnson *et al.*, 1948).

Additional work is necessary before the exact chemical mechanism of the urethan inhibition of division in marine eggs can be established. Moreover, it would be desirable to know whether similar reactions are responsible for the inhibition of cell division in mammalian tissues and tumors.

SUMMARY

1. Dilute solutions of urethan (about 1%) prevent the division of fertilized *Chaetopterus* eggs when the eggs are treated beginning 5 minutes after insemination.

Relative cytoplasmic viscosity determinations show that the mitotic gelation, which immediately precedes the formation of the mitotic apparatus, does not take place. In concentrations of urethan insufficient to prevent division (less than 0.5%) the mitotic gelation occurs normally. The inhibition is reversible since the eggs reassemble the mitotic apparatus and divide following return to normal sea water. Upon return to sea water the inhibited eggs divide directly into the number of cells present in the controls at that time, thus indicating that metabolic work and chromosome duplication are not affected. During this period there is a rapid and sharp increase in relative cytoplasmic viscosity. Eggs that have begun to form cleavage furrows are not able to complete cytokinesis upon exposure to urethan. The rigidity of the cortex is reduced by inhibitory concentrations of urethan. Cytological preparations reveal that urethan prevents the formation of the mitotic apparatus, and also destroys the organized structure of the mitotic apparatus after it has been formed.

2. Few of the eggs in 1% urethan divide until about four or five hours after fertilization. Untreated eggs at 21°C. divide by 58 minutes after insemination. The addition of small quantities of histamine, histidine, trypsin, chymotrypsin or lipase enables the eggs to divide sooner than four to five hours in the presence of urethan. Calcium, potassium, arginine and protamine were not effective.

3. It has been proposed that the urethan effects observed in this investigation may be reflections of an interaction with the hydrogen bonds that are believed to link together the macromolecular components of the mitotic apparatus.

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A BLOOD GROUP SYSTEM FOR SPINY DOGFISH, SQUALUS ACANTHIAS L.

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Recent expansion of interest in fish immunogenetics, particularly in blood group antigens (Cushing and Durall, 1957; Ridgway, Cushing and Durall, 1958; Suzuki, Shimizu and Morio, 1958; Sindermann and Mairs, 1959; Ridgway and Klontz, 1960), has resulted in description of antigen systems for tuna and herring, and in quantitative information about antigen frequencies in populations of herring, tuna and salmon. The elasmobranch fishes have not been examined thus far, but it seems quite possible that serological methods of population and migration study could be of particular value to investigations of certain shark species. The recognition and description of blood group antigens, and where possible the genetic systems that determine their occurrence, are preludes to quantitative determinations of antigen and gene frequencies in populations. As an exploratory step in such studies of sharks, and as part of a general immunogenetic investigation of marine organisms, this report is concerned with isoagglutinins and erythrocyte antigens of the spiny dogfish, *Squalus acanthias* L. Included are serological approaches and data supporting the proposal of a first blood group system—the "S" system—for this species.

MATERIALS AND METHODS

Dogfish were obtained from several places in the Gulf of Maine (Georges Bank, Casco Bay, Sheepscot Bay and Muscongus Bay) and were bled by puncturing the heart or by severing the caudal artery. Serum was decanted after overnight expression at 4° C. and inactivated at 50° C. for 30 minutes to destroy complement. Cells were washed three times in 1.5% saline and used in approximately 4% suspensions in 1.5% saline. Cross-reactions among dogfish sera and cells were tested by tube agglutination with .2 ml. undiluted serum and .05 ml. cell suspension. Tubes were read after 30-minute incubation at room temperature. Presence and degree of agglutination were recorded conventionally with the following symbols: ++++, complete agglutination with a single consolidated pellet; +++, strong agglutination with several large clumps; ++, moderate agglutination with numerous smaller clumps; +, weak agglutination with many very small clumps visible macroscopically; −, no agglutination. Saline controls were used throughout, and critical tests repeated. Absorptions of dogfish sera—which may remove certain antibodies selectively—involved addition of undiluted serum from one fish to packed washed cells from another fish in 1 : 1 proportions. The cell-serum sus-

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pension was incubated 10 minutes at room temperature, centrifuged, and the absorbed serum decanted and used in cell agglutination tests. One such absorption was sufficient to remove all antibodies reactive with the absorbing cells but in some cases left antibodies for cells from other fish.

Rabbit antisera were produced by 6 subcutaneous injections of 1 ml. washed dogfish cells given on alternate days. The rabbits were bled terminally 10 days after the last injection, and the antisera frozen in 3-ml. aliquots until used. Antisera were inactivated at 56° C. for 30 minutes just before testing, to destroy complement. Tests by tube agglutination used .2 ml. diluted antiserum and .05 ml. cell suspension. Readings were taken after 15-minute incubation at room temperature and 30-second centrifugation. Presence and degree of agglutination were recorded as described above. Antiserum absorptions, which constitute a fractionation method to produce specific reagents, involved addition of antiserum (diluted 1:2 with 1.5% saline) to packed washed cells from individual dogfish in proportions of 1 part cells to 4 parts antiserum. The cell-antiserum suspension was incubated 10 minutes at room temperature, centrifuged, and the absorbed antiserum decanted and tested at 1:4 dilution. One such absorption was sufficient to remove all antibodies reacting with the absorbing cells but in some cases left antibodies capable of reacting with cells from other individuals.

Dogfish donors and recipients used in isoimmunizations were tagged and maintained in sea-water tanks of the U. S. Bureau of Commercial Fisheries Biological Laboratory at Boothbay Harbor. The fish were trial bled by heart puncture; cross-reactions and tests with rabbit antisera were carried out on the samples obtained. Results of these tests determined the nature of the subsequent immunizations attempted. Recipient dogfish received 2 ml. of cells bled from the donor weekly and washed three times before injections, which were alternately intraperitoneal and intracardial. Trial bleedings for isoimmune antibodies were made two weeks following the fourth injection.

Dogfish embryos (15–23 cm.) used in the comparative study of erythrocyte antigens were obtained by dissections of gravid females or were aborted by such females after capture. They were bled by severing the caudal artery or by heart incision and tested with the same methods used for adults.

RESULTS

1. *Isoagglutination*

Cross-matching of cells and undiluted sera from 20 individuals gave the first clear indication of individual antigenic differences in dogfish, as seen in Table I. Sera from fish numbered 5, 6, 9, 12, 15, 16 and 17 contained isoantibodies (isoagglutinins) that reacted with cells of fish numbered 1, 2, 3, 4, 7, 8, 10, 11, 13, 14, 18, 19 and 20. Sera from fish whose cells were agglutinated did not contain antibodies for any of the cells tested. Conversely, cells from fish whose serum contained the agglutinins did not possess antigens reacting with any of the sera tested. The average isoagglutinin titer of the sera tested was 4.

The simplest explanation for the observed results was that the positive cells contained an antigen (tentatively labelled "antigen S") which negative cells lacked and that agglutinating sera contained a specific "anti-S" agglutinin for the antigen.

TABLE I

Cross-reactions of sera and cells from 20 dogfish

Cells / Serum	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	+++	++++	+++	+++	−	−	++	+++	−	++++	+++	−	++++	+++	−	−	−	+++	++++	++++
6	+++	++++	+++	++++	−	−	+++	+++	−	++++	+++	−	+++	+++	−	−	−	+++	++++	++++
7	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
8	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
9	++	+++	+++	+++	−	−	++++	++	−	+++	+++	−	+++	+++	−	−	−	++	+++	++++
10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
11	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
12	+++	+++	++++	+++	−	−	+++	+++	−	+++	+++	−	+++	+++	−	−	−	+++	+++	+++
13	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
14	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
15	+++	++++	+++	++	−	−	++++	+++	−	+++	+++	−	++++	++	−	−	−	+++	++++	++++
16	+++	+++	++++	+++	−	−	++++	+++	−	+++	+++	−	++++	++	−	−	−	+++	+++	++++
17	+++	+++	+++	+++	−	−	++++	+++	−	++++	+++	−	++++	+++	−	−	−	+++	+++	+++
18	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
19	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Validity of this explanation was supported by absorptions of sera containing anti-S agglutinins with positive and negative cells, and by an absorption analysis of a single serum, as illustrated in Table II.

Absorptions with cells lacking antigen S did not remove any antibodies, while absorptions with cells carrying antigen S removed all antibodies, not only for themselves, but for all other positive cells as well. This is strong evidence that the antigens involved are identical, or at least very nearly so. Sera containing the anti-S agglutinin were pooled as a test reagent (Reagent 1) for quantitative studies of S-antigen frequencies, as were sera lacking the anti-S agglutinin (Reagent 2). All subsequent samples were tested with the reagents and were also cross-matched in groups of 10 or 20, since it was not possible to keep cells for longer than one week without freezing or other treatment.

TABLE II

Results of absorption of dogfish isoantibodies by dogfish cells

Serum	Absorbed with cells of	Cells tested									
		1	2	5	6	9	16	17	18	19	20
5	1	−	−	−	−	−	−	−	−	−	−
5	2	−	−	−	−	−	−	−	−	−	−
5	5	+++	+++	−	−	−	−	−	+++	++++	+++
5	6	+++	++++	−	−	−	−	−	+++	+++	+++
5	9	+++	+++	−	−	−	−	−	++++	++	+++
5	16	+++	+++	−	−	−	−	−	+++	+++	++++
5	17	+++	++	−	−	−	−	−	++	+++	+++
5	18	−	−	−	−	−	−	−	−	−	−
5	19	−	−	−	−	−	−	−	−	−	−
5	20	−	−	−	−	−	−	−	−	−	−
6	5	+++	++++	−	−	−	−	−	++	+++	+++
6	1	−	−	−	−	−	−	−	−	−	−
9	2	−	−	−	−	−	−	−	−	−	−
12	9	+++	+++	−	−	−	−	−	++	+++	+++
15	17	+++	++++	−	−	−	−	−	+++	++++	++
16	18	−	−	−	−	−	−	−	−	−	−
17	5	+++	++	−	−	−	−	−	+++	++++	++++
18	2	−	−	−	−	−	−	−	−	−	−
18	16	−	−	−	−	−	−	−	−	−	−

2. *Antiserum absorptions*

Further elucidation of the antigen system disclosed by isoagglutination was obtained by absorption analysis of a rabbit anti-dogfish serum (BHD64R). The rabbit had been immunized with washed cells from a single dogfish found in preliminary tests to possess antigen S. The antibody content of the normal serum of this rabbit before immunization was not determined, but the titer of the unabsorbed antiserum was 128. Results of absorptions of this antiserum with cells of the same dogfish used in the isoagglutination study are illustrated in Table III.

Absorption with cells of 5, 6, 9 and 12, already found to lack reactive antigens in cross-matching tests, removed species antibodies—those which indiscriminately agglutinate cells of all individuals—but left antibodies which agglutinated cells of 1, 2, 3, 4, 7, 8, 10 and 11. Results of tests with the absorbed antiserum agreed with those obtained in the isoagglutinin study, cells of individuals 1, 2, 3, 4, 7, 8, 10, and 11 carrying an antigen not occurring on cells of individuals 5, 6, 9, and 12. The absorptions further disclosed a second antigen on the cells of fish number 2 that was not detected by isoantibodies in dogfish sera. Absorption of the rabbit antiserum with cells of dogfish number 2 removed antibodies for all other cells, while absorption with cells of 1, 3, 4, 7, 8, 10 and 11 removed antibodies for all others except those of fish number 2. For descriptive purposes the antigens detected by absorbed rabbit antiserum have been labelled "S_1" and "S_2"—S_1 being identical to the antigen detected by isoagglutination. Both antigens were reactive with the reagent prepared by pooling antiserum absorbed by cells of 5, 6, 9, and 12 (Reagent 3), and S_2 was reactive with the reagent prepared by pooling antiserum absorbed with cells of 1, 3, 4, 7, 8, 10 and 11 (Reagent 4). Antiserum absorbed with cells of fish number 2 constituted a negative control reagent (Reagent 5).

TABLE III

Results of absorptions of rabbit anti-dogfish serum by dogfish cells

Antiserum BHD64R absorbed with cells of dogfish:	Test cells											
	1	2	3	4	5	6	7	8	9	10	11	12
1	−	++	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−	−
3	−	++	−	−	−	−	−	−	−	−	−	−
4	−	++	−	−	−	−	−	−	−	−	−	−
5	+	++	+	+	−	−	+	+	−	+	+	−
6	+	++	+	+	−	−	+	+	−	+	+	−
7	−	++	−	−	−	−	−	−	−	−	−	−
8	−	++	−	−	−	−	−	−	−	−	−	−
9	+	++	+	+	−	−	+	+	−	+	+	−
10	−	++	−	−	−	−	−	−	−	−	−	−
11	−	++	−	−	−	−	−	−	−	−	−	−
12	+	++	+	+	−	−	+	+	−	+	+	−
Unabsorbed	+++	+++	+++	++++	++	++	++++	++++	+++	++++	+++	++

According to the reactions obtained, fish number 2 possessed antigens S_1 and S_2, fish number 1, 3, 4, 7, 8, 10 and 11 possessed antigen S_1, and fish number 5, 6, 9 and 12 lacked both antigens.

3. *Definition of reagents*

The results of isoagglutinations and antisera absorptions presented in previous sections show that it is possible to define five reagents for the detection of antigenic specificities S_1 and S_2.

(1) Dogfish serum containing anti-S agglutinins specific for S_1 antigen.
(2) Dogfish serum from S_1 positive fish lacking anti-S agglutinins—a negative control.
(3) Absorbed rabbit anti-dogfish serum (BHD64R) containing antibodies specific for S_1 and S_2 antigens.

TABLE IV

*Dogfish isoagglutination and antiserum absorptions**

Reagent:	Test cells									
	21 (S_0)	22 (S_1)	23 (S_1)	24 (S_0)	25 (S_1)	26 (S_1)	27 (S_1)	28 (S_2)	29 (S_1)	30 (S_1)
(1) Dogfish anti-S_1	–	++++	+++	–	++++	++++	+++	–	++++	++++
(2) Dogfish negative	–	–	–	–	–	–	–	–	–	–
(3) Rabbit anti-S_1 & S_2	–	+++	++	–	++	+	++	++	+	++
(4) Rabbit anti-S_2	–	–	–	–	–	–	–	++	–	–
(5) Rabbit negative	–	–	–	–	–	–	–	–	–	–
Serum of Dogfish No.										
21 (anti-S_1)	–	+++	+	–	++	++	+	–	++	+++
22 (–)	–	–	–	–	–	–	–	–	–	–
23 (–)	–	–	–	–	–	–	–	–	–	–
24 (anti-S_1)	–	++++	++	–	++	++	++++	–	++	++
25 (–)	–	–	–	–	–	–	–	–	–	–
26 (–)	–	–	–	–	–	–	–	–	–	–
27 (–)	–	–	–	–	–	–	–	–	–	–
28 (anti-S_1)	–	++++	++++	–	+++	++++	++++	–	++++	+++
29 (–)	–	–	–	–	–	–	–	–	–	–
30 (–)	–	–	–	–	–	–	–	–	–	–
Antiserum BHD64R absorbed with cells of dogfish										
21 (S_0)	–	+	++	–	+	++	+	+	++	+
22 (S_1)	–	–	–	–	–	–	–	++	–	–
23 (S_1)	–	–	–	–	–	–	–	++	–	–
24 (S_0)	–	+	+	–	+	+	+	++	+	+
25 (S_1)	–	–	–	–	–	–	–	++	–	–
26 (S_1)	–	–	–	–	–	–	–	++	–	–
27 (S_1)	–	–	–	–	–	–	–	+	–	–
28 (S_2)	–	+	++	–	++	++	++	–	++	+
29 (S_1)	–	–	–	–	–	–	–	+	–	–
30 (S_1)	–	–	–	–	–	–	–	+	–	–
Unabsorbed	++	++++	+++	+++	++++	++++	+++	++++	+++	+++

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* Erythrocyte antigens of each fish, as disclosed by this analysis, are in parentheses after each test cell number.

(4) Absorbed rabbit anti-dogfish serum (BHD64R) containing antibodies specific for S_2 antigen.

(5) Absorbed rabbit anti-dogfish serum (BHD64R) lacking antibodies for S_1 and S_2—a negative control.

The reality of the two antigens in the system and the specificity of the reagents for their detection were clearly demonstrated with a second series of samples tested first with the five reagents, then examined for isoagglutination, and finally analyzed by antiserum absorption, as seen in Table IV.

From this analysis, dogfish 22, 23, 25, 26, 27, 29 and 30 possessed S_1 antigen, 28 possessed S_2 antigen, 21 and 24 lacked either antigen, and none had both antigens. The antiserum absorptions provided a sixth reagent (absorbed with S_2 cells of 28) that was specific for cells containing S_1 antigen—completing the battery of reagents prepared from antiserum BHD64R necessary for detection of both S_1 and S_2 in quantitative studies of antigen frequencies. Subsequent samples, totalling 138, were tested with all six reagents. Dogfish 28 was the only one found to carry only antigen S_2.

4. *Isoimmunization attempts*

A further analysis of the erythrocyte antigens of individual dogfish was attempted with isoimmunization—injecting the cells of one fish into another fish. Dogfish in the series 21 to 30 described in the previous section and already characterized according to S antigens, were tagged and maintained in the sea-water tanks of the U. S. Bureau of Commercial Fisheries Biological Laboratory at Boothbay Harbor and were used in isoimmunization attempts, according to the following experimental design:

(1) dogfish 21, which lacked S_1 antigen and contained isoagglutinins for S_1, was injected with washed cells of 30 which possessed S_1;

(2) dogfish 25, which possessed S_1 antigen and lacked isoagglutinins for S_1, was injected with cells of 22, which possessed S_1; and

(3) dogfish 26 which possessed S_1 antigen and lacked isoagglutinins for S_1, was injected with cells of 24, which lacked S_1.

Two weeks after the final injection, sera from the three recipient dogfish were tested against cells of donors, and against cells of other dogfish in the series 21 to 30.

No change in agglutinin titer or specificity of dogfish sera was found after the isoimmunization attempts. Only the reactions of antigen S_1 were tested, since the single S_2 dogfish (number 28) died shortly after the first test bleeding. Under the conditions of the experiment (four weekly injections, tests two weeks after the last injection, sea-water temperature 12–17° C.) injection of S_1 cells into a recipient lacking S_1 caused no increase in anti-S_1 agglutinin titer. Injection of S_0 cells into a S_1 individual did not produce anti-S_0 agglutinins. Further isoimmunizations should be attempted with dogfish possessing S_2 and other antigens, possibly extending over a greater period of time or involving more frequent injections.

5. *Comparison of erythrocyte antigens of gravid females and embryos*

Since the spiny dogfish is an ovoviviparous species, it was possible to obtain presumptive genetic information about the S blood groups by comparing erythrocyte antigens of females and their well-developed embryos ("pups"). Detectable isoantibodies were not present in sera of nearly full-term pups, but erythrocyte antigens—agglutinated by isoantibodies in sera from adult dogfish and by absorbed antiserum—gave reactions of the same specificity and intensity as those of adult dogfish. Blood groups of females and their progeny are presented in Table V. Females with blood group S_1 had S_1S_2, S_1 and S_0 pups, while females with blood group S_0 had S_0 and S_1 pups.

TABLE V

Blood groups of gravid female dogfish and their unborn progeny

Blood group of mother	Blood group of embryos
(1) S_1	S_1, S_1, S_1, S_1, S_0
(2) S_0	S_1, S_1, S_1, S_1, S_1
(3) S_1	S_1S_2, S_0, S_0
(4) S_0	S_1, S_1, S_1, S_1
(5) S_1	S_1, S_1, S_1
(6) S_0	S_1, S_0, S_0, S_0
(7) S_1	S_1, S_1, S_1, S_1, S_0
(8) S_0	S_1, S_1, S_0, S_0, S_0, S_0, S_0, S_0
(9) S_1	S_1, S_1, S_1, S_1
(10) S_0	S_0, S_0, S_0, S_0, S_0
(11) S_1	S_1, S_1
(12) S_1	S_1, S_1, S_1, S_1, S_1, S_0

6. *The S blood group system of dogfish*

Although further genetic evidence—preferably based on results of known crosses—is necessary, the S blood group system can be proposed tentatively. Based on (1) results of isoagglutinations, antiserum absorptions and isoimmunization attempts; (2) comparisons of maternal blood groups with those of unborn pups; and (3) analyses of 138 individual dogfish blood samples from different Gulf of Maine areas with the six reagents described earlier, the following statements can be made:

(1) The S blood group system of spiny dogfish, *Squalus acanthias,* contains at least two antigens, S_1 and S_2, that may or may not be possessed by individual fish.

(2) Isoantibodies present in sera of most dogfish whose cells lack S_1 antigen will agglutinate cells possessing only S_1. Isoagglutinins for S_2 antigens have not been detected.

(3) Antigen S_1 can be demonstrated with antibodies remaining in rabbit antiserum after absorption by cells of dogfish containing S_2 antigen, and antigen S_2 with antibodies remaining after absorption by S_1 cells. Cells not agglutinated by either absorbed antiserum have been designated S_0. Thus far an anti-S_0 serum has not been found.

(4) Individual fish may possess both, either, or neither of the antigens, so possible blood groups (types) are S_1S_2, S_1, S_2, and S_0.

(5) Of the 138 dogfish tested thus far from the Gulf of Maine, two individuals had S_1S_2 blood group, 92 had S_1, 1 had S_2 and 43 had S_0.

DISCUSSION

The present paper demonstrates individual differences in erythrocyte antigens and an isoantibody system in a species of the elasmobranch fishes. A clearly defined antigen system, demonstrable with isoagglutinins and absorbed rabbit antiserum, exists in spiny dogfish. The system parallels the Tg system of tuna (Suzuki, Shimizu and Morio, 1958) in that it is composed of two antigens, with individuals possessing either, both or neither antigen. Similarities also exist with an isoagglutinin system described by Cushing and Durall (1957) in brown bullheads (*Ictalurus n. nebulosus*). The dogfish blood groups described here seem definitive enough in their present state to be used in quantitative studies of populations and in tracing migrations of infraspecies groups, if geographic variations in antigen frequencies exist. Thus far six individuals have been found that lacked S antigens and also lacked detectable isoagglutinins in their serum. This might be expected, since the agglutinin titers of individual sera varied considerably. As greater numbers of samples are tested, exceptions may occur, as they have been found to occur in human and other animal groups (Owen, 1954; Stormont, 1955), but the proposed system has proved remarkably specific and definitive thus far in tests of *Squalus* from the Gulf of Maine. Future studies may suggest modification of the system, just as extended studies of human blood group systems have resulted in modifications of original hypotheses (Landsteiner, 1901; von Dungern and Hirzsfeld, 1911; Friedenreich, 1936), but the reality of the dogfish S antigens described here seems clear.

Blood group systems are essentially genetic systems, and hence cannot be fully described, understood or accepted without genetic information. Any postulated classification of individual antigenic differences—such as that here proposed for dogfish—should be supported eventually by genetic evidence. It is difficult to make experimental crosses and to rear offspring of most marine fish for such studies, but the ovoviviparous species including *Squalus* can provide some interim data. Comparing blood groups of pregnant females and their unborn offspring may offer presumptive genetic evidence supporting erection of blood group systems. Such a comparison of female dogfish and their pups in the present work suggests that the S system of blood group antigens may be controlled by three alleles: S^1, S^2 and S^0. According to this explanation the S_1S_2 phenotype would have S^1S^2 genotype; S_1 would have S^1S^1 or S^1S^0; S_2 would have S^2S^2 or S^2S^0; and S_0 would have S^0S^0. Based on a three-allele hypothesis, quantitative data from the limited sample of adult dogfish presented in this paper result in the following gene frequencies: S^1—0.44, S^2—0.01, S^0—0.55. The number of individuals tested is small, so expected ideal values for the entire Gulf of Maine population may not be represented. Also, there may be local differences in frequencies, so the next logical step would be determination of antigen and gene frequencies in large samples of *Squalus* from different geographic areas. In addition to testing the genetic hypothesis, this quantitative work could provide some information about the degree of reproductive isolation of groups and the subpopulation structure of the species.

The existence of sharp individual differences in the antigenic constitution of dogfish offers the promise of comparable differences in other elasmobranch fishes. Since certain of the larger shark species are receiving increasing attention from biologists as well as from the popular press (Gilbert, 1960), immunogenetic methods may offer a useful approach to studies of their migrations and populations. While the large sharks provide a practical stimulus for research, smaller selachians such as dogfish may actually be animals of choice in such investigations. In addition to the possession of a clearly defined isoantibody system, dogfish occur in schools, are easily handled in marine aquaria, and yield copious amounts of blood. Also, since they are ovoviviparous, some genetic information may be acquired by comparing erythrocyte antigens of females and their pups. Future research in this laboratory will extend the comparative studies already reported, will include quantitative sampling for S antigen frequencies in different geographic areas, and will extend to other elasmobranch species—particularly the larger sharks.

SUMMARY

1. An erythrocyte antigen system of dogfish, *Squalus acanthias,* has been recognized and described with a progression of serological procedures including isoagglutination, antiserum absorption and isoimmunization. This system, the "S" blood group system, contains at least two antigens, "S_1" and "S_2." Individual fish may possess both, either or neither of the antigens, being of blood groups (types) S_1S_2, S_1, S_2 or S_0, respectively. Reagents specific for the antigens have been prepared from normal dogfish sera and absorbed rabbit antiserum, for quantitative studies of blood group frequencies in dogfish from different geographic areas. Fish sampled thus far in the Gulf of Maine (138 individuals) have the following frequencies: S_1S_2—1.4%, S_1—66.7%, S_2—0.7%, and S_0—31.2%. Blood groups of females and unborn pups are compatible with a simple genetic hypothesis of three alleles, S^1, S^2 and S^0, controlling the system.

2. The definitive nature of this system in *Squalus* promises potentially profitable immunogenetic investigations of other elasmobranchs, including the larger shark species. Blood group frequencies can be used in determining the degree of reproductive isolation of populations and may provide information about migrations of fishes.

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IONIC GRADIENTS IN SOME INVERTEBRATE SPERMATOZOA[1]

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While the variety of male gametes seems almost infinite, the vast majority of animals produce a spermatozoan of fairly typical structure consisting of a head, midpiece and tail (Retzius, 1910). The indications from many different sources are that these three regions (plus other specialized areas such as the acrosome) have highly specific functions. The midpiece, with its mitochondrial structure, is implicated in general metabolism, the head is heavy with genetic information via DNA, and the tail functions as a locomotor organelle to propel the information unit towards its ultimate repository, *i.e.,* the ovum or death (*cf.* Mann, 1949).

Compartmentalized as it is in neat morphological packages, the typical spermatozoan becomes a tempting material with which to attempt to identify various bits of chemical machinery ubiquitous in all cells. A fairly extensive body of literature exists on the chemical and metabolic structure of the head and, to a lesser extent, on the midpiece. Isolated tail fractions have been shown to contain special fibrous proteins which may contribute the contractile properties (*cf.* Nelson, 1959), some of these components being arranged according to a precise numerology that awaits final elucidation (*cf.* Serra, 1960).

One of the most characteristic features of living cells, neglecting for the moment bacteria, etc., is the existence of an ionic gradient $K_i > K_o$ and, for most cells $Na_o > Na_i$ (Steinbach, 1952). These gradients are generally associated with irritability of cells and have at times been discussed as a reflection, at least, of an available free energy gradient (Fleckenstein, 1954). Ionic gradients have not been studied in any detail in sperm cells and, so far as we are aware, no previous attempt has been made to determine whether the production and/or the maintenance of an ionic gradient is a function of the whole structure or only a part thereof. The present report presents preliminary data on the distribution and exchangeability of Na and K of whole sperm and of separated head-midpiece fractions and tails. The head-midpiece complex, for the sperm of three species tested, appears capable of maintaining good ionic gradients. Tails, separated from the rest of the complex, appear to contain Na, K and Cl in free diffusion equilibrium with the environment.

MATERIAL AND METHODS

Mature sperm were collected as follows:

1. Male *Arbacia punctulata* were induced to shed into sea water by electrical stimulation. The suspensions were then diluted appropriately and used as described below.

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2. *Mytilus edulis* sperm were collected by mincing the gonads in sea water, straining the resultant brei through cheesecloth and then subjecting to mild centrifugation to remove heavier contaminants. Microscopic examination showed these preparations to be reasonably free of tissue fragments.
3. *Phascolosoma gouldi* sperm were collected by draining body fluids of mature males into sea water and removing the large cell components by mild centrifuging. Clean suspensions of sperm could be obtained easily in this fashion.

The general pattern of treatment of sperm suspensions was as follows: The suspensions, maintained at sea-water table temperature (18–22° C.), were adjusted to the desired chemical composition. Separation of head-midpieces from tails was accomplished by 5–10 seconds at high speed in a Servall Blender. This method gave suspensions that could be separated by 10 minutes' centrifuging at ca. 2000 G into a pellet of apparently tail-less head-midpiece structures and a cloudy supernatant of filamentous tails. Especially in Mytilus, the tail suspension fibers frequently aggregated into sheaves.

Tails were collected as pellets by centrifuging the supernatant of the low speed centrifugation for 10 minutes at 15,000 G in a Servall superspeed centrifuge. This resulted in a clear supernatant apparently devoid of formed elements.

Concentration of cells was adjusted so that 10 ml. of suspension of whole sperm yielded on the order of 100–200 mg. of packed pellet as determined by weighing previously tared centrifuge tubes. Rather than attempt extensive cell counts, estimates of trapped fluid in the pellets were made by adding either C^{14} inulin to the suspension or $Na_2S^{35}O_4$ thus enabling the determination of inulin or sulphate "spaces." As noted below, there is certainly some question as to whether this is a true measure of a morphological volume of extracellular or cellular space, but the method seems more meaningful than the highly variable cell count method of determining cell volume. With fractions of such varying chemical composition it was deemed unwise to attempt to express Na or K contents per unit of nitrogen or of dry weight. Thus concentrations are expressed as millimols per 1000 grams of wet pellet or as figures derived from these on the basis of inulin spaces and/or dry weights.

Na and K were measured on extracts of pellets or on supernatant fluids with the Coleman flame photometer. Chlorides were frequently measured on the same solutions with an Aminco-Cotlove chloride meter. Extracts were prepared by heating briefly the suspended pellet in 10 ml. of water with 2 drops of glacial acetic acid added. Digestion with HNO_3 of residues from the above treatment showed almost complete removal of Na, K and Cl from the suspended material.

C^{14} was counted in the usual manner with a thin window gas flow counter.

RESULTS

Table I summarizes the results of the first series of observations on the basic composition of the three types of sperm. Figures for bull and human sperm, uncorrected for inulin spaces, are given for comparison. Inulin spaces of the pellets are high and there are reasons to believe that the inulin is penetrating into some portions of the cell mass. However, inulin space is not greatly variable with time and is used in calculating cell water concentrations. Several points need emphasis. K is concentrated in the sperm as it is in other cellular systems. If cell water volume could be truly represented as total pellet volume minus dry weight and inulin space, the K concentration of cell water is very high indeed for Arbacia and Phascolosoma.

Na and Cl contents are variable, as will be apparent also from subsequent tables. Table I indicates that no chloride is present in the non-inulin space. On the contrary, the fact that inulin space frequently exceeds chloride space of the pellet is an indication that inulin penetrates to some extent within the morphological boundaries of the sperm.

TABLE I

Dry weights and inulin spaces (as % of wet pellet) and concentrations as mM./1000 grams of wet pellets. Derived concentrations (mM./L. cell water) calculated assuming 97% of dry weight of pellet to be in sperm and external medium of composition Na = 410, Cl = 500, K = 10 mM. Composition of bull and human sperm from Cragle et al., 1958 and Keitel and Jones, 1956.

	% Dry (pellet)	Inulin %	% Dry (sperm)	Na		Cl		K	
				Pellet	Cell$_{H_2O}$	Pellet	Cell$_{H_2O}$	Pellet	Cell$_{H_2O}$
Arbacia (2)	24	46	45	195	23	150	—	136	440
Mytilus (4)	27	26	36	166	107	161	—	78	145
Phascolosoma (8)	18	44	32	209	76	216	—	115	300
Bull				76				62	
Human			16	101		28		39	

It is known that sperm of such forms as Arbacia and Mytilus can survive, properly diluted in sea water, for periods of several hours. Table II shows that these sperm can also maintain their ionic gradients for extended periods of time. With Mytilus there is a decrease of pellet size with time. In the absence of counts of viable cells, it is impossible to tell whether this involves an average decrease in cell size or a disintegration of some fraction of the populations. Whichever the cause, the decrease takes place within the first hour to hour and a half, pellet size being constant thereafter.

Potassium in sperm is nearly completely exchangeable (Fig. 1) at a fairly rapid rate. Half-time for exchange of cellular K with K^{42} of the medium is on the order of three hours for Mytilus, 1.5 hours for Phascolosoma, and less than an hour for Arbacia.

Figure 1 shows that the time course of exchange of K^{42} added to the medium with K of the pellet follows the usual course of an initial rapid exchange (extracellular?) followed by a second slower component. There is, however, no indication that any significant amount of K is sequestered within the sperm or otherwise made inaccessible to free exchange. The exchange of Na^{24} with Na of sperm is very rapid. Accurate results have not been obtained but the half-time for exchange is distinctly less than 30 minutes for all three types of sperm.

Table III lists average values for the analyses of head-midpiece and tail fractions as compared to whole sperm from the same species. The values listed

TABLE II

Pellet concentrations (mM./1000 gm. wet) of Na, Cl and K for samples of a suspension centrifuged at the times indicated. Sperm suspensions stored on sea water table at 20° C.

Time minutes	Weight of pellet %	Conc. mM./Kg.		
		Na	Cl	K
Mytilus				
10	100	162	126	74
30	98	159	164	75
90	91	155	151	67
210	90	155	139	64
Arbacia				
20	100	193	287	110
130	101	168	267	88
300	100	174	232	99

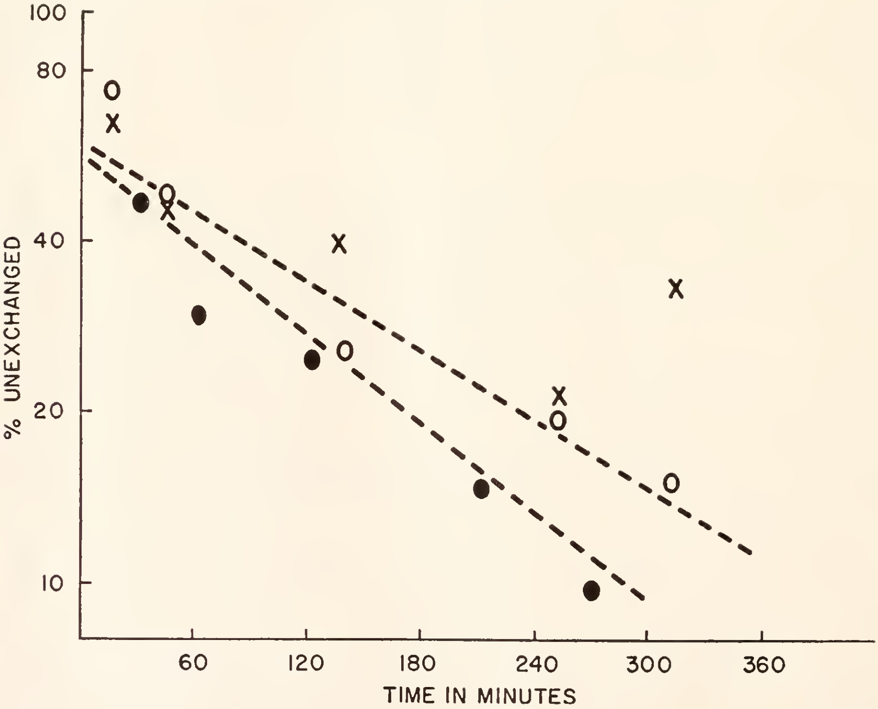

FIGURE 1. Time course of exchange of K^{42}, added to suspension fluid at zero time, with K of Mytilus sperm. Figures uncorrected for inulin space or dry weight. Dots and circles represent separate experiments; crosses are results from one run with isolated Mytilus sperm head-midpieces.

in Table III were obtained from analyses in which samples of whole sperm, head-midpiece and tail were taken from the same batches and processed during coincident time periods. Weights given are pellet weights obtained by weighing the previously tared centrifuge tubes after the supernatant fluids had been decanted, followed by inverting the tubes on filter paper for about five minutes. The gain in weight due to similar treatment of centrifuge tubes containing only pure water (or the supernatant from the centrifugation of whole sperm suspension) is in the range 10–15 milligrams (5–10% of the usual pellet weight).

With Arbacia and Mytilus sperm, head-midpieces and tail pellets contained less K and more Na and Cl than did the whole sperm, inulin spaces increasing markedly. Phascolosoma sperm heads, on the contrary, had a higher K content with lower Na and Cl, inulin spaces remaining about the same. Tail fractions of all three forms had very high inulin spaces, high Na and Cl contents and low K.

TABLE III

Relative weights, inulin spaces and Na, Cl and K concentrations of whole sperm, head-midpieces and tail pellets. Units as in Table I.

	Relative weight	Inulin space %	Na	Cl	K
Whole sperm					
Arbacia (4)	100	41	191	200	119
Mytilus (2)	100	39	244	271	44
Phascolosoma (8)	100	44	202	206	106
Heads-midpieces					
Arbacia (4)	72	56	305	329	54
Mytilus (2)	96	52	302	353	31
Phascolosoma (8)	64	44	173	192	130
Tails					
Arbacia (4)	33	85	394	401	15
Mytilus (2)	21	68	356	373	10
Phascolosoma (8)	20	92	422	384	25

Pellet weights of head and tail fractions corrected for the drainage factor (10 mg.) added together should approximate the corrected pellet weight of the whole sperm, since comparable volumes were centrifuged, starting with a standard suspension. This summation holds reasonably well for Arbacia and Phascolosoma. Mytilus sperm heads swell and hence the pellet weights of the parts add to more than the pellet weight of the whole sperm.

While head fractions undoubtedly were contaminated to a slight degree by separated tails, it is probable that the pellet weights indicate minimum values for weights of sperm tails as compared to the whole. On this basis tails make up 20–30% of the total sperm weight.

The increase in inulin space for the pellets of head fractions of Arbacia and Mytilus sperm as compared to whole sperm could represent either a general increase in inulin penetration into all units or a complete destruction of some of the units. It is impossible to select, on any rigorous basis, between these two alternatives since no method was available to us to distinguish, by independent criteria, dead heads from live heads. However, much of the increase in Na and Cl and decrease of K could be accounted for by assuming that the increase in inulin space represented complete physiological destruction of the appropriate number of morphologically intact heads. Phascolosoma sperm would then be assumed to have tougher heads, all surviving the homogenization of the sperm suspension. Head fraction pellets of Arbacia and Mytilus sperm maintain relatively constant ionic contents over periods of several hours, thus showing that, if partial injury is responsible for the decreased K contents, the effects are not progressive. On the whole, we suggest that homogenization results in destruction of some units of the head suspension, the K content of individual intact heads remaining at at least as high a level as that of intact sperm.

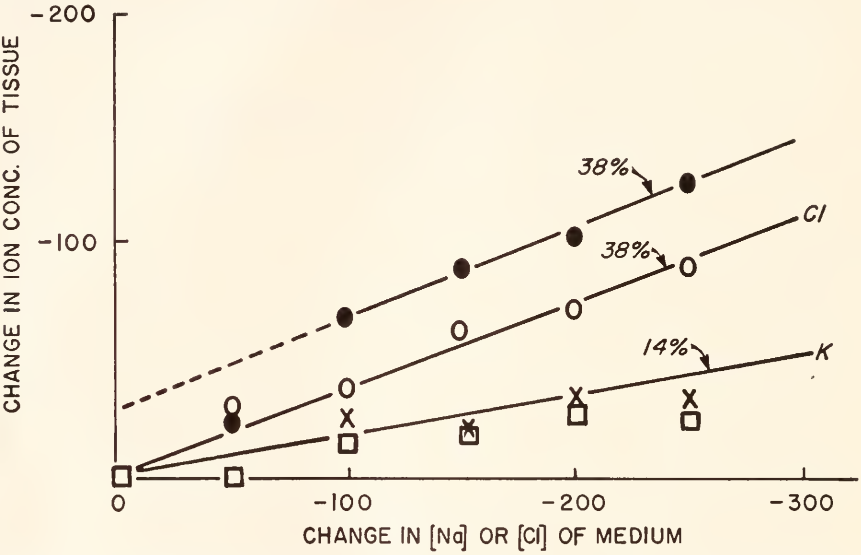

FIGURE 2. Values for Na (●), K (x), Cl (○) and pellet weights (□) of Arbacia sperm suspended 30 minutes in sea water diluted with 10 mM. KCl. Units in mM. per liter or per Kg. wet weight of pellet. The greatest dilution was a 50:50 mixture of sea water and 10 mM. KCl. Figures at arrows represent slopes of curves.

When Arbacia sperm are treated for 30 minutes with mixtures of sea water and 10 mM. KCl, thus varying Na_o, Cl_o and total ionic strength but holding K_o constant, the cells swell slightly as indicated by the increase in pellet weight (Fig. 2). In approximately 50% sea water the weight increase is slightly over 20%. Using the usual methods of calculation this would indicate a "non-osmotic" space (b value) of nearly 80%. Thus there is no evidence for an ordinary osmotic behavior of the sperm cells. As the medium is diluted, K_o holds constant, K_i decreases in somewhat greater degree than can be accounted for by the increase in cell volume. Pellet Na_i and Cl_i decrease with decrease in Na_o and Cl_o but in a manner indicating that the change, in the greater dilutions, is primarily in the inulin space fraction.

Arbacia treated with various concentrations of KCl in sea water (Na_o held constant, K_o and Cl_o varied) show a remarkably constant Na_i concentration (Table

TABLE IV

Arbacia sperm exposed to ionic concentrations indicated, prepared by adding KCl to sea water. Cell water concentrations calculated assuming 35% inulin space, 40% dry weight of sperm.

Na			$\frac{Na_i}{Na_o}$	Cl			$\frac{Cl_o}{Cl_i}$	K			$\frac{K_i}{K_o}$
Medium	Tissue	$Cell_{H_2O}$		Medium	Tissue	$Cell_{H_2O}$		Medium	Tissue	$Cell_{H_2O}$	
420	218	180	0.43	510	218	102	5.0	10	83	205	20.5
420	218	180	0.43	610	303	230	2.6	110	176	352	3.2
420	218	180	0.43	710	375	375	2.2	210	202	338	1.6
420	201	138	0.33	810	435	390	2.1	310	269	409	1.3
420	222	191	0.45	910	535	550	1.6	410	335	490	1.2
420	233	220	0.52	1010	650	760	1.3	510	405	580	1.1

IV). K_i and Cl_i increase markedly with no evidence of the reciprocal loss of Na_i noted for such systems as the frog sartorius. Based on calculated cell water concentrations, K_i/K_o is over 20 and Cl_o/Cl_i is 5 in normal sea water. Both ratios approach unity as total external ionic strength approaches two times that of sea water. There is no significant change in volume as total ionic strength is varied until very high concentrations are reached.

Mytilus sperm, treated in a similar fashion, show some increase in Na_i, Na_o being held constant (Table V). Both K_i and Cl_i increase somewhat but especially K_i is regulated to change much less than K_o, whereas K_i/K_o in normal sea water is about 15, the ratio is 0.5 in sea water made half molar with KCl.

In view of the interest in the use of glycerol as protective agent in freezing sperm, Mytilus sperm were suspended in 13% (by volume) of glycerol in sea water, controls being suspended in sea water diluted in corresponding fashion with distilled water. Little volume change was noted in 4–5 hours. K_i decreased with time in both glycerol-treated sperm and controls. Glycerol-treated sperm lost K and gained Na and Cl during the first half hour of treatment, the difference then being maintained. Motility in glycerol was maintained in an odd vibratile fashion, motility in the controls appeared normal throughout the experiment.

TABLE V

Mytilus sperm suspended 30 minutes in solutions of KCl in sea water as indicated. Calculations of cell H_2O concentrations assuming 40% dry weight of sperm and using inulin spaces indicated. K_i/K_o ratios calculated using cell water concentrations.

Relative volume pellet	% Inulin pellet	Na			$\frac{Na_i}{Na_o}$	Cl			$\frac{Cl_o}{Cl_i}$	K			$\frac{K_i}{K_o}$
		Medium	Tissue	$Cell_{H_2O}$		Medium	Tissue	$Cell_{H_2O}$		Medium	Tissue	$Cell_{H_2O}$	
100	32	420	197	155	0.37	510	197	83	6.1	10	66	153	15.3
98	33	420	205	166	0.39	610	257	163	3.7	110	127	226	2.1
98	32	420	202	166	0.39	710	313	210	3.4	210	171	255	1.2
102	34	420	232	225	0.53	810	404	325	2.5	310	225	301	0.9
109	41	420	266	265	0.63	910	483	310	2.9	410	266	274	0.7
106	42	420	275	285	0.68	1010	555	375	3.1	510	310	278	0.5

DISCUSSION

While the results reported here must be regarded as a part of a preliminary survey, certain conclusions may be drawn. In common with all cells studied, spermatozoa of the type studied maintain high K and low Na and Cl concentrations as compared to their normal active environment. In mammals this ionic differentiation becomes pronounced only after the sperm descend to the lower portions of the reproductive tract or are released. In this case the ionic gradients are probably established due to changes in the seminal fluid, not in the sperm themselves (Salisbury, 1956). The precise environments during formation and maturation of sperm of the invertebrates used in the present studies are not known to us.

As the ionic environment is altered, there is evidence that minimum portions of Na_i and K_i are held rather firmly, K_i being considerably higher than Na_i. There is no indication of a reciprocal relationship between Na_i and K_i nor is there any equality of the Donnan Ratios K_i/K_o and Cl_o/Cl_i except under very abnormal conditions (0.5 *M* KCl in sea water).

Both K_i and Na_i are rapidly and nearly completely exchanged with the environment, thus showing that ion selection does not involve a rigid sequestering of the elements. Some evidence for active extrusion mechanisms might be obtained from more precise flux measurements but no evidence for the process can be adduced from the present data. Rather, sperm behave as though they could retain relatively fixed amounts of Na and K, meanwhile allowing exchanges freely between medium and cell. In general the electrolytes of sperm behave rather similarly to those of certain smooth muscles from invertebrates (*cf.* Steinbach, 1940).

With respect to the problem of localization of the ion distribution mechanisms, these results are suggestive but by no means conclusive. It is certain that the head-midpieces of all three types of sperm retain K and continue to exclude Na and Cl with reference to the medium. With Phascolosoma, head-midpiece fractions maintain higher respective ionic gradients than do whole sperm. With Arbacia and Mytilus the gradients of head-midpiece fractions are reduced. While there is no compelling evidence, it is tentatively assumed that mechanical damage to portions of the head-midpiece fractions of Arbacia and Mytilus is responsible for the decrease in magnitude of Na_o/Na_i, K_i/K_o and Cl_o/Cl_i in the head pellets as compared to whole sperm pellets.

Tail fractions always showed very high inulin spaces and little if any ability to maintain ion gradients. It is suggested that the contractile organelle of the spermatozoan is entirely dependent on the ion-concentrating mechanisms of the head-midpiece portion for its ionic gradients, assuming such exist *in vivo*. On the basis of the known structures and metabolic activities it would be logical to look for evidence that the midpieces play major roles in the processes. Mitochondria in general are known to be able to retain selectively various ions.

Isolated sperm tails contain enzymes usually associated with contractile processes and also show spontaneous movements on application of ATP. These spontaneous movements are best described as twitchings rather than conducted waves as seen in normal attached tails. It seems possible that normal coordinated activity of sperm may depend upon ionic gradients imposed on the contractile tails by the metabolic activity of the midpiece regions.

The sperm used in these experiments were all of the simple type presumably possessing short midpieces and tails with the 9 + 2 arrangement of fibrils (Afzelius, 1955). It is anticipated that comparative studies with mammalian and other sperm with longer midpieces and more complicated flagellar structure, indicating a possible extension of mitochondrial components, will shed further light on the compartmentalization of ionic gradients.

Summary

1. The concentrations of Na, K and Cl of pellets of sedimented sperm of Arbacia, Mytilus and Phascolosoma have been determined. With the aid of inulin space determinations on the pellets, it can be shown that sperm resemble other cells studied in maintaining, relative to the sea water environment, high internal K concentrations and low Na and Cl.

2. Sperm of the three species were separated into head-midpieces and tail fractions. The former retain the ionic selection properties of the whole sperm. Isolated tails appear freely diffusible to all constituents including inulin.

3. Using radioactive tracers it is shown that Na and K of sperm are freely exchangeable with the environment.

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SOME OBSERVATIONS ON THE GROWTH RATE OF SEA URCHINS IN THE GENUS STRONGYLOCENTROTUS[1]

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While vast amounts of basic biological information have been derived from studies using the eggs and early embryos of sea urchins, so little is known concerning the growth rate, age, and longevity of the adults that both Harvey (1956) and Comfort (1956) have remarked on the scarcity of information. Shearer, de Morgan and Fuchs (1914), Elmhirst (1922), Aiyar (1935), Moore (1935), Bull (1938), Nataf (1954), and Lewis (1958) have contributed important information for species in a number of different genera. Despite the fact that the green sea urchin *Strongylocentrotus droebachiensis* (O. F. Müller) occurs in suitable habitats throughout much of the northern half of the northern hemisphere and in places is one of the most conspicuous and numerous macroscopic members of the marine benthos and intertidal fauna, data on its growth rate are few. Soot-Ryen (1924) cautiously presented a few data based on relatively small collections from Ramfjorde on the Norwegian coast and clearly indicated his doubt as to the justification of defining year-classes. He also pointed out that (p. 16) "*Var. pallida*, G. O. Sars, lebt mit *f. typica* zusammen." Thus, in view of Vasseur's (1951, 1952) findings, it would appear that Soot-Ryen was dealing with a collection including two species. Schorygin (1928) presented size-frequency distributions based on large collections from the Barents Sea, but his separating *S. droebachiensis var. atroviolaceus* from what he considered the typical form of the species suggests that his data were derived from the study of *S. pallidus* (G. O. Sars). Grieg (1928) presented data on size-frequency distributions for both the Folden Fjord and for Bals Fjord but made no mention of the existence of more than one form or species in the genus. Some of his collections were dredged from depths as great as 200 meters, and Vasseur (1951, 1952) found *S. pallidus* to be the commoner species along the Norwegian coast in deeper dredgings. Thus it appears likely that he was dealing with both species without distinguishing between them. The present writer (Swan, 1958) has reported the size-frequency distribution of a collection of *S. droebachiensis* made intertidally in York, Maine. There appears to be no doubt that these belong to the species considered by Vasseur (1951) as *S. droebachiensis,* but with the existence of two species of *Strongylocentrotus* at Dröbak (Vasseur, 1952) Mortensen's (1943, p. 214) statement concerning Müller's type to the effect that "the fact that it is polyporous is a definite proof of the correctness of identifying the common N. Atlantic species with it, this being the only polyporous Echinoid of the N. Atlantic region" becomes a weak argument. Madsen's (1959) figure and discussion of this type specimen make it appear likely that determining its identity with either species would be nearly if not quite impossible.

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On the west coast of the United States *S. purpuratus* (Stimpson) is probably the sea urchin most used for embryological, physiological, and biochemical studies, and in Japan other members of the genus and the closely related *Hemicentrotus pulcherrimus* (A. Agassiz) are extensively used for these purposes. To the writer's knowledge there are no available data pertaining to the growth rates of any of these species. It is the purpose of the present work to begin to fill these gaps.

Experiments and Observations at New Castle, New Hampshire, and York, Maine

On the morning low tides of June 1 and 3, 1958, sea urchins (*S. droebachiensis*) were collected from near the southeast corner of the island on which Nubble Light off Cape Neddick is located (at 43° 09′ 04″N and 70° 35′31″W in the township of York, Maine). Except for the time they were actually being measured, the animals were kept refrigerated until evening of the same day, when they were placed in cages (Swan, 1960) submerged in a large tide pool (about 140 × 60 feet with a surface water level about one foot above mean low water and a maximum depth of about three feet at tide levels below that of the pool) at New Castle, N. H. (43° 3-2/3′N, 70°42-4/5′W). The place of collection was the locality studied earlier (Swan, 1958). Only those specimens approximating 8–10, 24–26, 40–42, or 50–52 mm. in test diameter were collected. Between their collection and their deposit in the cages, each specimen was re-measured, and those not within the desired size ranges were discarded. Three cages were used, and the animals of one of the three larger size groups were placed in each. The 8–10 mm. individuals were divided among four one-pint polyethylene containers, perforated with several holes (3–5 mm. in diameter) through the cover and bottom. Then the containers were pressed into holes in the cross-pieces of the cages.

The tide pool naturally supports a moderate population of sea urchins; is well protected from heavy surf by ledges; is near kelp beds, which furnished food for the caged animals; and is behind a private estate and thus free from much interference from the public. The cages were securely anchored to the bottom of the pool where its depth at low water was about 1½ feet. When the animals were placed in them, several nearly entire healthy plants of the kelp *Laminaria digitata* (Lamouroux) and/or similar-appearing species were added to each cage, and pieces of the blades were placed in the plastic boxes in such manner that they would not obstruct the passage of water. Subsequently, until the experiments were terminated on June 3, 1959, the cages were visited and the kelp was renewed at intervals ranging from 6 to 18 days. When the experiment was ended, the animals were killed in formalin in sea water and after 24 hours dried at room temperature. Their spines were brushed off, and the tests were measured with vernier calipers. The results of this experiment are summarized in Table I.

The almost perfect viability is notable. The single specimen lost from the group of greatest diameter died within the first month. Two others were accidentally crushed. In previous experiments of this type there was some evidence of disease as a possible cause of mortality. It appears to be important to observe the following practices: (1) keeping the animals in good condition between the time of their collection and their return to the ocean, (2) feeding only fresh living kelp, and (3) keeping the cages clean of anything that might impair the free passage of water through them.

The sizes of animals at the beginning of the experiment were selected largely on the basis of size-frequency modes found or suspected in the earlier study (Swan, 1958).

One of the factors that might be expected to affect growth rate is the material utilized as food. Earlier experiments confirm this. On June 12 and 13, 1956, 129 of the smallest individuals that could be found were collected from the Nubble. These were divided into five groups having approximately the same distribution of sizes. One group was immediately preserved and dried. The other four groups were put in one-pint polyethylene containers, two of which were supplied with *Laminaria* and two with *Ascophyllum nodosum* (L.) Le Jolis. These were all placed in a cage in the tide pool at New Castle. On June 26 one of the containers with each alga and its urchins was taken to Boothbay Harbor, Maine, where through the courtesy of the U. S. Fish and Wildlife Service and the Maine Dept. of Sea and Shore Fisheries the writer was able to suspend them in a cage from their common dock. Before the end of the first month mortality had reduced each of the groups at Boothbay Harbor to 20 specimens and those at New Castle to 18 with the *Ascophyllum* and 17 with the *Laminaria*. On August 23, the living specimens at Boothbay Harbor were measured as were those at New Castle two days later. Those fed *Ascophyllum* at Boothbay Harbor averaged 6.9 mm. in diameter whereas those at New Castle averaged 7.4 mm. With *Laminaria* those at Boothbay Harbor averaged 8.2 mm. and those at New Castle 8.9 mm. For numerous reasons these figures and the implied amounts of growth cannot be considered accurate, but they do suggest that there is probably a difference in growth rates between localities even when the supplied food is the same. On September 13 all the animals at Boothbay Harbor were found dead, apparently as a result of plugging of the holes in the polyethylene containers.

TABLE I

Data on the growth of Strongylocentrotus droebachiensis *(O. F. Müller) held in cages at New Castle, N. H. from June 1 or 3, 1958, to June 3, 1959*

Original		Concluding			
			Test diameters (mm.)		
Test diam. (mm.)	#	#	Range	Mean	Standard deviation
8–10	41	40	23.1–31.6	27.5	±2.2
24–26	42	41	33.4–45.3	40.2	±2.8
40–42	39	38	45.3–54.1	49.8	±2.1
50–52	16	15	51.4–57.3	55.5	±1.7

On June 11, 1957, nearly a year after the initiation of this experiment, 16 animals with each kind of food were still living at New Castle. These were preserved and dried. The dried specimens are shown in Figure 1, and the data obtained are given

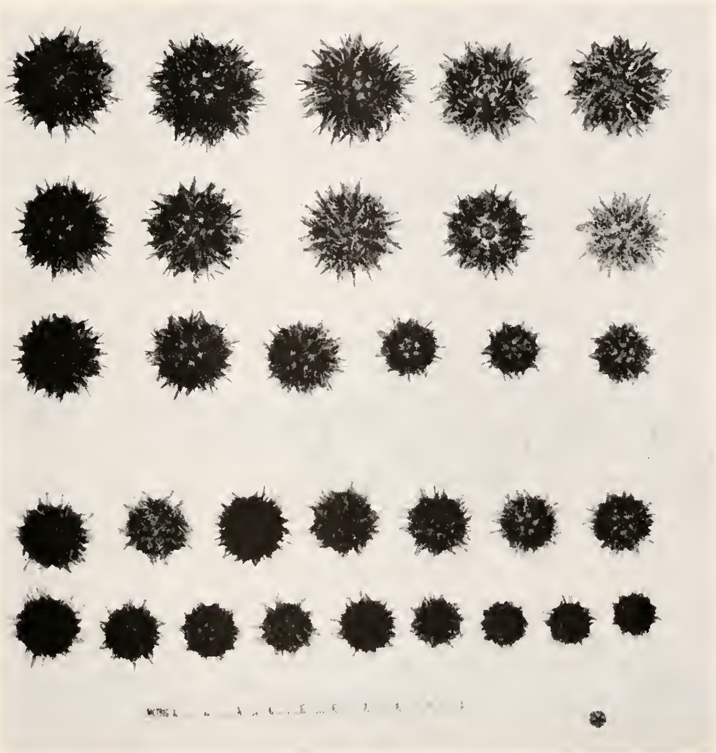

FIGURE 1. Effect of differences in food over a period of one year on the growth rate of *S. droebachiensis* (O. F. Müller) held in plastic containers at New Castle, N. H. Upper three rows, fed *Laminaria.* Lower two rows, fed *Ascophyllum.* Lower right, specimen as originally collected. (Photograph by George M. Moore.) Rule length = 10 cm.

in Table II. From these figures it appears that the increase in mean diameter of of the specimens fed *Laminaria* exceeded that of those fed *Ascophyllum* by more than 70%. This should not be interpreted as meaning that *Laminaria* is more nutritious. While no quantitative study was made, it appeared that much more of the *Laminaria* was eaten.

TABLE II

Data on growth of Strongylocentrotus droebachiensis *supplied with two different algae and held in cages at New Castle, N. H. from June 12 or 13, 1956, to June 11, 1957*

	Numbers		Diameters (mm.)		
	Original	Preserved	Range	Mean	σ
Basic controls preserved immediately	25	25	3.5–6.1	5.0	±0.63
Those fed *Ascophyllum*	27	16	11.8–18.6	15.4	±2.07
Those fed *Laminaria*	27	16	14.7–27.7	22.1	±4.08

On June 11 and 12, 1957, another series of experiments was begun, but by November 4, mortality was so high that these were terminated. The following data were recorded. (1) 16 of 17 small *S. droebachiensis* (initially 7–10 mm.) lived in perforated polyethylene containers with no food added for four months. After that many died, but so did many others supplied with various algae and kept nearby. (2) Small *S. droebachiensis* eat and grow well when supplied *Corallina officinalis* L. or *Chondrus crispus* (L.) Stackhouse, but these tend to break or to be chewed so that after a few days there are many small pieces in the containers. These pieces tend to clog the holes and thus impede water circulation. For feeding larger animals in the cages, much of either of these algae was soon lost through the wire mesh.

TABLE III

Data on growth of Strongylocentrotus *held in cages at Friday Harbor, Washington, in 1959 and 1960*

Start of experiment				Conclusion of experiment			
Date 1959	#	Diameters (mm.)		Date 1960	#	Diameters (mm.)	
		Range	Mean			Range	Mean
S. droebachiensis							
July 6	12	26–32	ca. 30	July 5	11	47.2–59.7	52.8
Aug. 12	10	55–57	ca. 56	Aug. 8	9	57.8–66.6	62.1
Aug. 11	4	76–78	ca. 76½	Aug. 8	1	77.2	—
S. echinoides							
July 26	17	25–27	ca. 26	July 26	15	36.3–44.3	40.6
July 26	20	41–43	ca. 42	July 26	14	44.5–53.6	48.6
July 26	8	53–57	ca. 55	July 26	7	54.1–60.7	57.1
S. franciscanus							
July 6	15	26½–31	ca. 29	July 5	15	45.0–59.3	54.2
July 6	15	46½–52½	ca. 49	July 5	14	56.9–67.2	62.0
S. purpuratus							
July 8	2	11 & 19	15.0	July 5	2	23.4–39.9	31.7
July 16–20	12	24–28	ca. 26	July 18	10	37.0–48.2	42.6

EXPERIMENTS AND OBSERVATIONS AT FRIDAY HARBOR, WASHINGTON

While the writer was at the Friday Harbor Laboratories of the University of Washington, numerous courtesies were extended to him by Dr. R. L. Fernald, the Director of the Laboratories, and other members of the staff. Thus he was able to collect an assortment of sea urchins belonging to the genus *Strongylocentrotus* and to suspend some of these in cages from the Laboratories' dock for observations on growth rate. All urchins caged for study at Friday Harbor were fed the large kelp, *Nereocystis luetkeana* (Mertens) Postels and Ruprecht. A summary of the data so obtained is given in Table III.

From the data obtained on *S. droebachiensis,* even though they are rather small in amount, it appears quite obvious that at Friday Harbor with a diet of *Nereo-*

cystis this species grows both more rapidly and to a larger size than it does at New Castle, N. H., with a diet of *Laminaria*. Whether this is a result of differences in the food, differences in other environmental factors, genetic differences between the populations, or to some combination of these remains unanswered.

S. echinoides A. Agassiz and H. L. Clark (which, as suggested by Vasseur (1952), is closely allied to or possibly even identical with *S. pallidus*) grew at a decidedly different and slower rate for animals of comparable sizes.

Limitations in cage space prevented studies with the long-spined and often very large *S. franciscanus* (A. Agassiz) except in small sizes. The data obtained from these latter suggest that this species in its smaller sizes grows a little more rapidly than does *S. droebachiensis* under comparable conditions. This suggests that the large size often attained by individuals of the species is largely the result of continuing growth rather than from more rapid early growth.

The few data obtained for *S. purpuratus* (Stimpson) indicate a relatively slow growth rate, comparable with that of *S. echinoides* in the smaller sizes; but the large sizes attained by the former species in this region (Swan, 1953) indicate that its growth must continue with less reduction in rate for a longer time.

Discussion

On June 29, 1953, a considerable number of sea urchins ranging from about ½ to 1½ mm. in test diameter were collected at the "Nubble." These appear to have been the young of the year. When the collection was made at the same place four years later (Swan, 1958) no such small animals were found, but well-defined modes in the size-frequency distribution were found at 8–10 and 24–26 mm., and possible modes 40–42 and 52–54 mm. Thus when the 8–10 mm. animals in the present experiments grew to a mean diameter of 27.5 mm. in a year, we have close agreement with the 24–26 mm. diameter for the next year class. Likewise the growth of the 24–26 mm. animals to a mean diameter of 40.1 mm. confirms the 40–42 mm. mode for the next year class. The fact that the 40–42 mm. animals attained a mean diameter of only 49.8 mm. in the year may suggest that the previously suspected mode at 52–54 mm. is too high, and a re-examination of the earlier size-frequency distribution (Swan, 1958) suggests the possibility of a mode between 40–42 and 52–54, possibly at about 46 mm. However, at these sizes and ages overlaps in age groups become great and numbers of individuals small. Thus extreme care in interpretation is required. That the group initially 50–52 mm. in diameter attained a mean diameter of 55.5 mm. is perhaps notable, as is the near lack of mortality in this group. Only three of the 1305 individuals studied earlier exceeded 54 mm. in diameter. Of the factors that could account for this continued life and growth the following should be considered: (1) the caged animals were not subject to the collection for food by man; (2) the cages may have afforded protection against other predators and against some physical environmental factors (as wave action and moving stones); (3) *Laminaria* for food was always available; and (4) there may be unrecognized differences between conditions at the "Nubble" and those of the tidepool that affect growth. In this latter respect surf action may affect available feeding time. At the "Nubble" the urchins may be severely beaten by waves as often as four times a day, whereas at the tide pool surf action is very slight.

The growth of these largest individuals suggests three more questions concerning the life of *Strongylocentrotus droebachiensis* in these localities: (1) What is the longevity of the animals? (2) Do they grow as long as they live? (3) Do they reproduce as long as they live?

For comparison of these findings with those of earlier works on "*S. droebachiensis,*" estimated diameters at various ages are listed in Table IV. The exact time in the year when the collections were made varies some, but all were from essentially summer work. In the different localities there may be differences in time of spawning and in the settling of the larvae; and as mentioned previously, the data of the earlier workers may well represent collections of *S. pallidus* or mixtures of it with *S. droebachiensis.* To take the estimates made on dredged material as proof of age at various sizes is going beyond justification, but a few observations may be worth noting. It appears probable that Soot-Ryen missed the class of the year and that his groups are listed a year too young. It also appears possible that Schorygin's estimates are displaced by a year and that his 12–20 mm. group is actually the II-year class. If these speculations are correct, the figures of the earlier workers are in amazingly close agreement with each other, and it took the animals they studied about five years to grow to the size reached by the animals studied on the northern New England coast in four.

TABLE IV

Sizes of "S. droebachiensis" *as estimated at various ages in millimeters of test diameter* (*Numbers without parentheses indicate size range in class; numbers in parentheses indicate estimate of modal size for the class*)

Age class	Swan N. H. & Me., U. S. A.	Soot-Ryen (1924) Ramfjorde, Norway	Schorygin (1928) Barents Sea	Grieg (1928) Folden and Bals Fjords, Norway
0	½–1½	6–10	—	½–2.5
I	(8–10)	12–22 (18)	12–20	(5–6)
II	(24–26)	22–33 (28)	21–31	(15)
III	(40–42)	33–40 (37–38)	32–41	(24 & 32)
IV	(46–54?)	43–52 (48)	42–52	(40?)
V		52–60	53–60	(50?)

The data obtained at Friday Harbor are so few that further comments are scarcely justified. It is quite obvious that in the smaller sizes the *S. droebachiensis* grown in cages there grew much more rapidly than in New Hampshire. In fact specimens grew from a size scarcely larger than those on the New Hampshire coast would be at two years to their size at four or more years in a single year. As mentioned earlier the factors involved in this rapid growth have not been determined. While it may be pure coincidence, the growth attained by the experimental *S. echinoides* in Friday Harbor almost exactly agrees with the earlier worker's findings for the growth rate of their "*S. droebachiensis*" on the northern coasts of Europe. Since *S. echinoides* is very close to *S. pallidus,* with which species they may have been dealing, at least in part, this observation may be significant.

SUMMARY

1. For certain areas of the New Hampshire and southern Maine coasts, evidence has been accumulating indicating that in June of the year of settling *S. droebachi-*

ensis have attained a size of only ½–1½ mm. in test diameter, and that the modes of subsequent year classes are approximately 8–10, 24–26, 40–42 and somewhere between 46 and 54 mm.

2. *S. droebachiensis* was found to grow considerably faster in test cages at Friday Harbor, Washington, but differences in the food supplied might be responsible, at least in part, for this difference.

3. Exploratory tests on growth rate were carried out at Friday Harbor on *S. echinoides*, *S. purpuratus*, and *S. franciscanus*.

4. Observations of earlier workers on growth rate in sea urchins of the genus *Strongylocentrotus* have been reviewed and compared with the present findings.

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